Development of novel drug screening assays and molecular characterization of rifampicin and pyrazinamide resistance in *Mycobacterium tuberculosis*

Von der Fakultät für Lebenswissenschaften

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Table of Contents

1 Introduction

Tuberculosis (TB) is the leading cause of mortality worldwide caused by any one bacterium. It is an important common contagious disease with public health problem in both the developed and developing countries. TB is caused by *Mycobacterium tuberculosis, a* pleomorphic, non-motile, non-spore forming acid-fast bacterium belonging to the family Mycobacteriaceae. It has unusually high GC content (65%) in its genomic DNA and also a very high lipid content in the cell wall, which accounts for more than 50% of dry weight of the cell (Barrera 2007). M. tuberculosis is a slow growing intracellular pathogen, requiring approximately 20 hours to replicate.

TB can be considered as an ancient disease which can be traced back to the ancient Egyptian civilization ca. 1550–1080 BC (Nerlich et al. 1997). A recent specimen found in Turkey of early evolving human was diagnosed with TB caused by *Leptomeningitis tuberculosa* (Kappelman et al. 2008) thus making the relation of TB with human back to 500,000 years.

Due to the improving living standard, health systems and hygienic condition, the prevalence of TB decreased in the 20th century. However this phase did not last long. Since the late 1980s an alarming global resurgence of TB has been observed, particularly in developing countries (Kochi 1991).

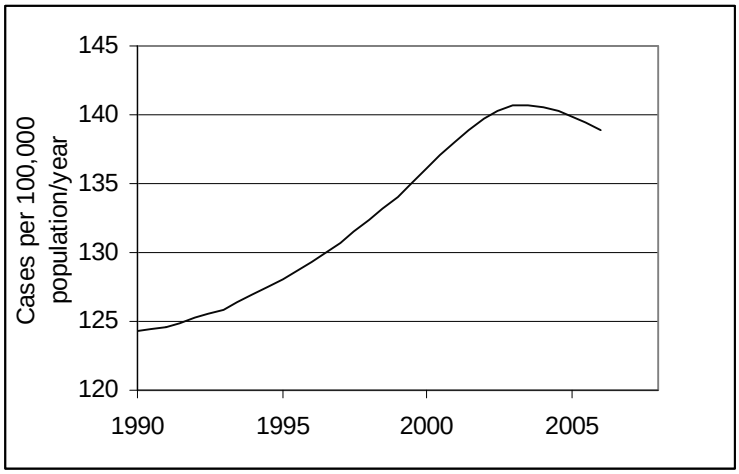


Fig. 1. Estimated global and incidence rate of tuberculosis, 1990–2006. Source: (WHO 2008)

One third of the human population is infected by TB, of whom 5-10% will develop active disease. The organism has the ability to stay in a dormant phase in host allowing asymptomatic infection that may persist for decade or life long. However, during the weakening immunity as is caused by malnutrition, old age or diseased condition e.g. in person infected with HIV (Human immunodeficiency virus), *M. tuberculosis* is reactivated and leads to active TB (Smith 2003). The TB epidemic situation is worsened by HIV pandemic with almost 13 million people currently co-infected with HIV and TB (Barnes et al. 2002).

Dormancy has been associated with non-replicating or very slow growth of *M. tuberculosis* that resides in granulomas, a heterogeneous assembly of macrophages, in the lungs of infected individuals. It is generally assumed that the microenvironment in the granulomas is characterized by hypoxia, nutrient starvation and reactive oxygen and nitrogen species (Wang 1991; Fenton and Vermeulen 1996; Zhang 2004).

1.1 Epidemiology of TB

Between 2-3 million people die from this disease alone and some 8-10 million new cases are reported every year (Porter 1991), which accounts for the total infected individuals to 1700 million. An estimated 1.7 billion people, i.e. one-third of the population, carries the causative agent of the disease and thus risking the development of disease whenever the immunity of individual weakens. The report regarding the incidence of TB worldwide has increased steadily from 1990 to 2004 from 124 million to 141 million cases.

Most of the deaths from TB is caused by the synergistic relationship of TB with HIV. As of 2006, 9.2 million new cases and 1.7 million deaths from TB were reported, of which 0.7 million cases and 0.2 million deaths were in HIV- positive people (WHO 2008).

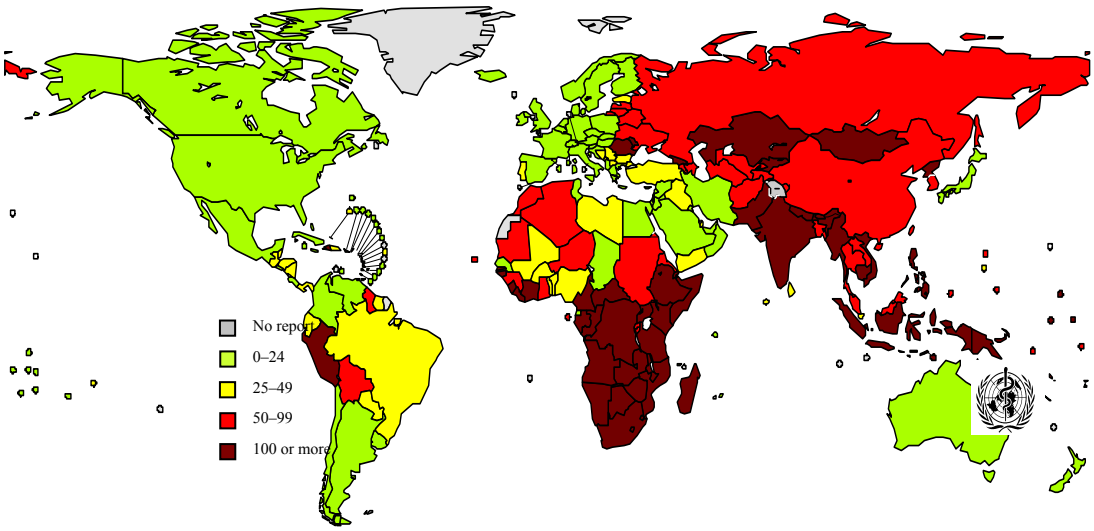


Fig. 2. Notified TB cases (new and relapse) per 100 000 population. Source: (WHO 2008)

1.2 Emergence of drug resistant TB

For TB therapy, 6-9 month of chemotherapy with first line anti-TB drug combinations of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (ETH) are prescribed.

However patients infected with Multiple Drug resistant TB (MDR-TB) require second line drugs, which have significantly more side effects to the patients. MDR-TB is resistant against two or more first line drugs. Recent trend of MDR-TB is rising which has led to an alarming situation and failurein treating MDR-TB. With the evolution of drug resistant organisms, the current chemotherapies are insufficient and novel therapies to deal with the rising resistant TB are needed. Until recently, drug development was in sluggish phase, due to technical limit, the size of potential market and due to the higher rate of the disease in the developing world (Copeland 2005).

The genetic basis of antibiotic resistance in clinical strains is predominantly attributed to mutations in specific genes or their promoter region. Extensive Drug Resistant or

Extreme Drug Resistance (XDR) tuberculosis can be defined as TB that is resistant to first- and second-line drugs. In other words it is resistant to both rifampicin and isoniazid and in addition resistant to any fluoroquinolones, and to at least one of three injectable second-line anti-tuberculosis drugs (capreomycin, kanamycin, and amikacin) used in the treatment of tuberculosis.

Increase in the incidence of XDR-TB has added more problems to the already serious TB epidemic.

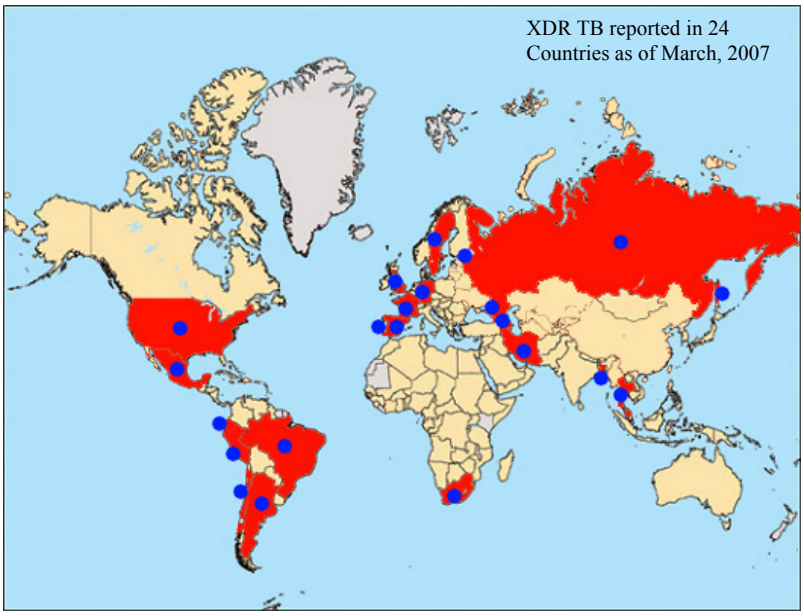


Fig. 3. Countries with confirmed cases of XDR-TB as of March 2007.

Until March 2007, XDR-TB was detected in 24 different countries (Fig.3). By February 2008 the number increased to 47 countries (WHO 2008). Just within a difference of one year, the reported XDR-TB cases increased in 23 countries, which is a clear indication of worldwide spread of XDR-TB.

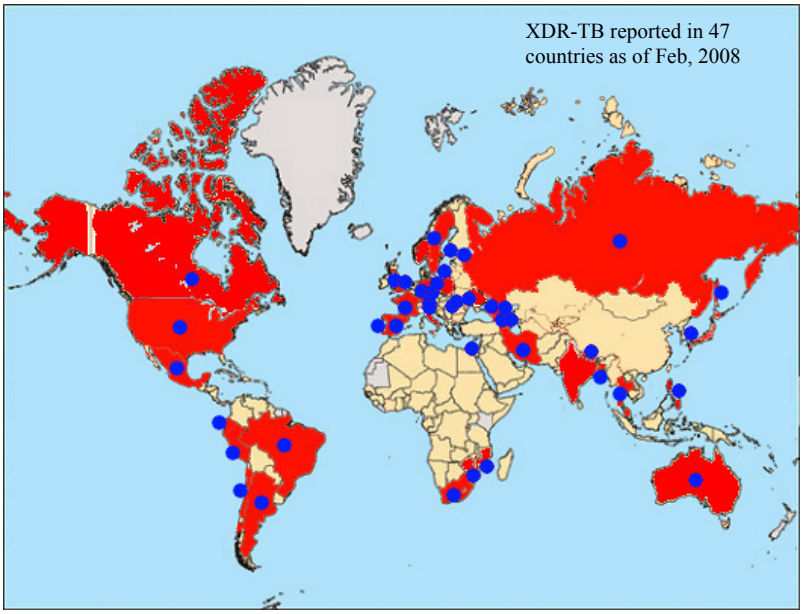


Fig. 4. Countries with reported cases of XDR-TB as of Feb 2008 increased up to 24.

1.3 Current therapies

Currently there are ten antituberculosis drugs approved by the United States Food and Drug Administration (FDA). Additionally, the fluoroquinolones, which are not approved by the FDA for TB, are also commonly used to treat TB caused by drug resistant strains or for patients who are intolerant to some of the first line of drugs.

The currently available antituberculosis agents and their modes of action are tabulated below.

Table 1. Antituberculosis agents, their mode of reaction and the target gene(s) of pathogen.

Antituberculosis Agent	Action of Inhibition	Target Gene
First line drugs		
Rifampicin	Bacterial RNA polymerase	*rpoB (Telenti et al. 1993)*
Isoniazid	Inhibitors of Cell Wall Synthesis. Inhibition of mycolic acids synthesis in *M.* tuberculosis (Winder et al. 1970; Winder and Collins 1970)	*katG* encoded catalase peroxidase (Heym and Cole 1992; Zhang et al. 1992; Cockerill et al. 1995; Heym et al. 1995; Pym et al. 2002) *inhA* encoded long chain enoyl-ACP reductase (Quemard et al. 1995) *ahpC* (Wilson and Collins 1996; Chen et al. 2005)
Pyrazinamide	Acidification of cytoplasm and de-enerizes membrane by hydrolyzing pyrazinamide (Konno et al. 1967; Butler and Kilburn 1983)	*pncA* (Scorpio et al. 1997)
Ethambutol	Inhibition of arabinogalactan synthesis, which is necessary for the construction of the outer envelope of the mycobacterial cell envelope (Sareen and Khuller 1990)	*embA*,*embB*, *embC* (Sreevatsan et al. 1997)

Second line drugs		
Fluoroquinolones (Levofloxacin, moxifloxacin, gattifloxacin, ciprofloxacin, ofloxacin)	Inhibition of DNA gyrase, an ATP-dependent Type II DNA topoisomerase that catalyzes the negative supercoiling of DNA (Wang 1991)	*gyr* (Wang 1991; Takiff et al. 1996) *lfrA* (Takiff et al. 1996)
Capreomycin	Inhibition of translation by affecting ribosomes	*rrs* (16S RNA) (Taniguchi et al. 1997; Suzuki et al. 1998) *tlyA* (Maus et al. 2005)
Cycloserine	Peptidogylcan synthesis	*alrA, gadA* y
Streptomycin and Related Aminoglycosides	Drug inactivation via acetylation (Benveniste and Davies 1973) However, this mechanism of resistance has not been reported in *M. tuberculosis*.	*rpsL* gene (Finken et al. 1993; Nair et al. 1993; Meier et al. 1994)
Amikacin and Kanamycin	Inhibition of protein synthesis	*rrs* (16S RNA) (Maus et al. 2005)
Ethionamide	Inhibition of mycolic acids synthesis in *M. tuberculosis* (Winder et al. 1970; Winder and Collins 1970)	*katG* encoded catalase peroxidase (Heym and Cole 1992; Zhang et al. 1992; Cockerill et al. 1995; Pym et al. 2002) *inhA* encoded long chain enoyl-ACP reductase (Quemard et al. 1995) *ahpC* (Wilson and Collins 1996; Chen et al. 2005)
p-Aminosalicylic Acid	Inhibition of iron uptake, Folic acid biosynthesis (Rengarajan et al. 2004)	

1.4 Rifampicin

Rifampicin (Rif) was first introduced in 1972 as an antitubercular drug which was extremely effective against *M. tuberculosis*. It has since then proved as the most potent and the most effective drug against TB. It has minimum inhibitory concentration (MIC) of 0.1 µg to 0.2 µg (Woodley et al. 1972). It is a first-line drug for the treatment of all sorts of TB with known or presumed sensitivity to drug. It has an activity against organisms that are in log phase (Jindani et al. 1980) or semidormant bacterial population, thus accounting for its antibiotic activity (Dickinson and Mitchison 1981). It is a semi-synthetic antibiotic derived from rifamycin B which is a fermentation product of *Streptomyces mediterranei*. The structure of Rifampicin is shown in figure 5.

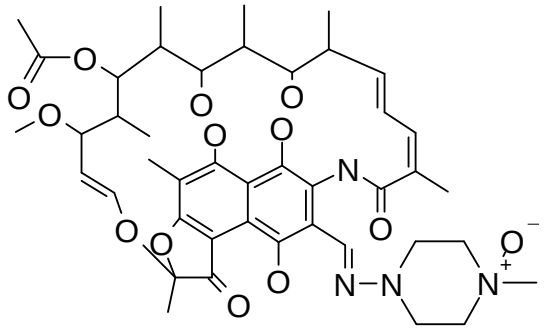


Fig. 5. Structure of Rifampicin

The DNA-dependant RNAP is one of the key enzyme in the central dogma of all living being. This enzyme synthesizes RNA copy from a DNA template during the process of gene transcription. The RNAP structures of *Thermus aquatus* and *E. coli* are known.

Rifampicin has a specific inhibitory action against bacterial RNA polymerase (RNAP) and little effect on human RNAP. The molecular basis of action of rifampicin has already been well characterized in *Escherichia coli* (Wehrli 1976; Jin and Gross 1988). Rifampicin binds in a pocket of the RNAP β subunit deep within the DNA/RNA channel and blocks the RNA exit pathway. As a result, RNAP bound to rifampicin is able to initiate RNA chain synthesis, but is unable to elongate the RNA product beyond a length of 2–3 nucleotides (Campbell et al. 2005). The vast majority of rifampicin resistant mutants harbor substitutions in RNAP β subunit residues that either make direct contacts with Rifampicin or are located near the binding pocket (Campbell et al. 2001). The mechanism of action of rifampicin in *Mycobacterium smegmatis*, a fast growing closely related species of *M. tuberculosis* has been studied in detail (White et al. 1971).

1.5 RNA polymerase of *M. tuberculosis* and drug resistance

The RNA polymerase of *M. tuberculosis* has been studied extensively since decades. It is a multi-subunit structure composed of several subunits like RpoA, RpoB, RpoC, and RpoZ and sigma unit. It is a relatively large molecule of about 363 kDa (Cole et al. 1998). The core enzyme consists of four subunits comprised of α2, β, and β', which catalyse the synthesis of RNA. The promoter-specific transcription initiation requires core enzyme together with σ subunit, which together makes up holoenzyme. In *M. tuberculosis* genome 13 putative σ factors are present (Cole et al. 1998) and most of those found to be associated with virulence (Smith 2003).

The α subunit of the RNAP with molecular mass of 37.7 kDa contains 374 amino acid, is encoded by *rpoA* gene, which is 1044 bp long. There are two subunits in RNAP. It helps in initiating the assembly of the enzyme and recognizes regulatory factors.

The β subunit is encoded by 1172 nt *rpoB* gene of *M. tuberculosis*. It is relatively large protein with molecular mass of 129 kDa and 1172 amino acids. It is the core unit of RNAP.

The β' subunit is the largest subunit with molecular mass of 146.7 kDa. It contains 1316 amino acids and is encoded by *rpoC* gene. It binds to DNA non-specifically.

The ω subunit is 11.8 kDa, encoded by *rpoZ* gene with 333 amino acids. Its function is not known in detail but it is assumed to restore denatured RNA polymerase to its functional form in vitro. It promotes assembly of RNAP.

As a result of mutation in the rpoB gene specific conformational changes result in defective binding of the drug (Jin and Gross 1988). At least 66 different kinds of mutation are already characterized which are related to the resistance against rifampicin (Jin and Gross 1988). Most mutations are restricted to a 69-bp region and are dominated by single nucleotide changes resulting in single amino acid substitutions as shown in Fig. 6.

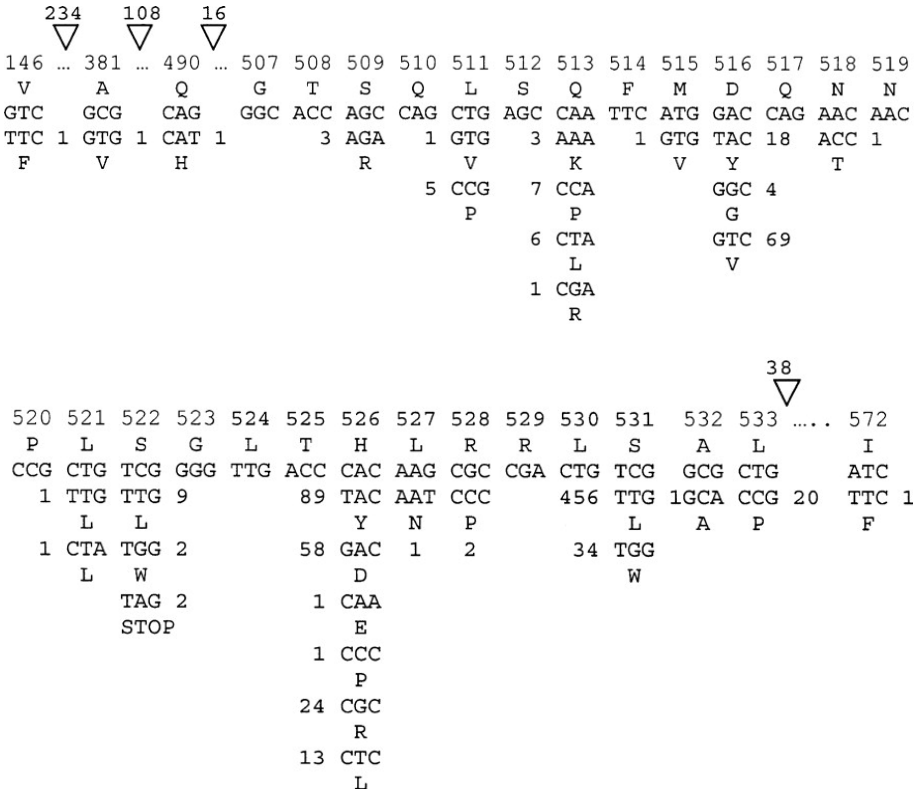


Fig. 6. Distribution of mutations within the *rpoB* gene in *M. tuberculosis*. The arrows and numbers indicate the number of codons that were omitted from the diagram as they had no mutation. Source: (O'Sullivan et al. 2005)

1.6 Pyrazinamide

Pyrazinamide (PZA), an analog of nicotinamide, is one of the most important drugs for anti-TB shortcourse chemotherapy, particularly it is the only drug which is bactericidal to dormant *M. tuberculosis* (Heifets and Lindholm-Levy 1992) and is effective in the acidic environment inside macrophages where M.tuberculosis primarily resides and survives (Dickinson and Mitchison 1970; Mitchison 1985). It is a prodrug for tuberculosis, which requires conversion to the bactericidal compound pyrazinoic acid (POA) by bacterial pyrazinamidase (PZase) activity (Blanchard 1996). The specific action of POA is not yet known, but it has been suggested that accumulation of POA results in a pH reduction leading to non-specific inhibitory effect on cellular metabolism (Boshoff et al. 2002). It has been shown that POA can disrupt *M. tuberculosis*

membrane potential, affecting the transport function at acidic environment (Zhang et al. 2003). The report by Ngo (Ngo et al. 2007) that PZA and POA were able to inhibit mycobacterial fatty acid synthase I (FASI) has not been confirmed and is controversial.

1.6.1 Pyrazinamidase and drug resistance

PZase is an amidase, which converts amide to an acid. In *M. tuberculosis* it is encoded by 561 nt *pncA* gene, which produces 19.6 kDa protein consisting of 186 amino acids.

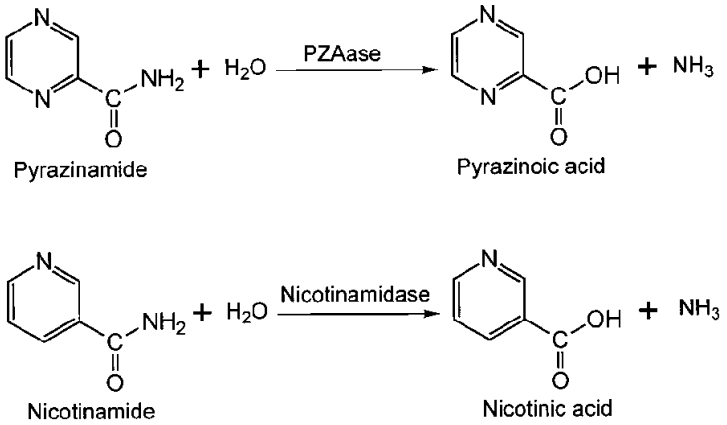


Fig. 7. Schematic representation of pyrazinamidase and nicotinamidase reaction.
Source: (Frothingham et al. 1996)

Mutations leading to a loss of PZase activity cause PZA resistance in *M. tuberculosis* (Konno et al. 1967; McClatchy et al. 1981; Butler and Kilburn 1983; Trivedi and Desai 1987; Miller et al. 1995). This is the major mechanism of PZA resistance in clinical isolates of *M. tuberculosis* (Scorpio et al. 1997; Sreevatsan et al. 1997; Lemaitre et al. 1999; Marttila et al. 1999; Cheng et al. 2000; Miyagi et al. 2004; Jureen et al. 2008; Mphahlele et al. 2008). However, all PZA resistant strains of *M. tuberculosis* do not contain mutation in *pncA*, suggesting presence of alternative mechanisms for PZA resistance. Different studies showed that 72-97% of PZA resistant *M. tuberculosis* harboured mutation in *pncA* gene or in its regulatory region (Sreevatsan et al. 1997;

Rodrigues Vde et al. 2005; Barco et al. 2006; Jureen et al. 2008). The crystal structure PZases of *P. horikoshii* has been solved (Fig. 8).

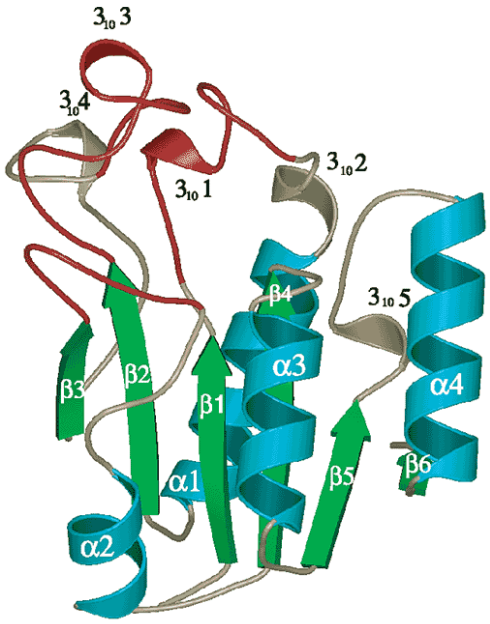


Fig. 8. Crystal structure of nicotinamidase of *Pyrococcus horikoshii* 999 (PDB ID: 1ILW). α-Helices and β strands are colour-coded in cyan and green, respectively. The three loops in red (from residues 10 to 21, 128 to 133, and 52 to 72, respectively) contribute most to the scaffold of the active site. Mutations that confer PZA-resistance show some degree of clustering in the corresponding regions of PZase of *M. tuberculosis*. (Du et al. 2001)

A comparaison of the PZAse of *M. tuberculosis*, *M. smegmatis* and *E. coli* shows that the active site of the PZase in *M. tuberculosis* probably lies in the 8-18, 49-71 and 96-102 amino acid residues PZase (Du et al. 2001).

In *M. smegmatis,* fast growing mycobacterium there are two known enzymes (PncA and PzaA) responsible for the amidase activity (Guo et al. 2000).

1.7 Summary

In summary, following are the main reasons for the global re-emergence of TB and the increasing prevalence of MDR and XDR TB:

1. Inadequate, inacurate and slow diagnosis of infection and TB disease by currently available means by sputum microscopy, culture, chest radiography and molecular means. This is also true for the diagnosis of resistant strains.

2. Incomplete protection by *M.bovis* BCG, the only vaccine used world-wide

3. Longe term treatment duration of TB therapy leading to non-compliance and hence emergence of MDR and XDR TB.

4. Inavailability of drugs to treat MDR and XDR TB

There is an urgent need for a better understanding of the mechanisms of resistance to antibiotics in *M. tuberculosis* and for the development of rapid assays suitable for high-throughput screening for the development of new drugs not only against MDR and XDR TB but also for the treatment of persistent and latent tuberculosis infection (LTBI).

1.8 Objectives of the study

The objectives of the study were to investigate drug resistance in clinical strains of *M. tuberculosis* and to evaluate currently available assays and/or to develop new assays suitable for drug development for resistant MDR and XDR strains.

More specifically, the objectives were:

1. Evaluate current assay for RNAP and PncA (PZAse).
2. Attempt to reconstitute *M. tuberculosis* RNAP holoenzyme for use in drug screening.
3. Screening of potential drug candidates using the optimized assay for RNAP.
4. Identify mutation in *pncA* gene which are responsible for Pyrazinamide resistance.

The work presented in this thesis was done independently but forms a part of large international research project on TB drug develeopment "PERSISTENT TB" and "NEWTBDRUGS" supported by the European Union (Coordinator: M. Singh, Braunschweig, Germany). These projects have focussed on an integrated strategy of drug development by structural analysis of novel targets, virtual and real screening based identification of leads, new organic synthetic chemistry and functional evaluation of best hits in in-vivo mouse model. Following identification of novel drug targets, 3-D structure of several targets (Tpx, NirA, CysK1, CysM, ALADH) validated by gene inactivation has been solved (Schnell et al. 2005; Stehr et al. 2006; Schnell et al. 2007; Agren et al. 2008) ;CysH; manuscript in preparation).

2 Materials and Methods

2.1 Chemicals and reagents

2.1.1 For routine lab work

Table 2. Chemicals used for routine lab work.

Chemicals	Company	Comments
Acetic Acid	Roth	SDS Gel Destaining
Acrylamide	Roth	SDS
ADC Enrichment	BD	Enrichment for Medium
Adenosine-5´-triphosphate-γ-(4-methylumbelliferyl) ester	Hannover University	Fluorescent RNAP Assay
Agar	Difco	Medium
Agarose	Roth	DNA Gel
Alkaline phosphatase	Fermentas	Fluorescent RNAP Assay
Ampicillin	Sigma Aldrich	Antibiotics
AMPSO	Fermentas	Fluorescent RNAP Assay
APS Super Broth	Otto Nord Wald	Protein Expression
Benchmark Protein Ladder	Biotage	Protein Standard
BioMix 500	Bioline	PCR Kit
BSA Standard Set	Bio-Rad	Protein Estimation
Buffer Solution pH=9.0 und 7.0	Roth	pH Calibration
Bug Buster	Merck Biosciences	Cell Disruption
Coomassie Brillantblue	Serva	SDS
Coomassie Plus Prot. Assay Reagent	Pierce	Protein Estimation
Crystal Screen	Hampton	Crystallization Trial
Crystal Screen 2	Hampton	Crystallization Trial
DTT	Sigma Aldrich	SDS
Ethambutol	Sigma-Aldrich	Antibtiotics
Ethanol	J. T. Baker	Various uses
GelStar Nucleic Acid Stain	Lonza Verviers	DNA Staining
GeneRuler™ 1 kb Plus DNA Ladder	Fermentas	DNA Ladder
GeneRuler™ 100 bp DNA Ladder	Fermentas	DNA Ladder
GeneRuler™ 1kb DNA Ladder	Fermentas	DNA Ladder
Glycerol	Roth	Various uses
Guanosine-5´-triphosphate-γ-(4-methylumbelliferyl	Hannover University	Fluorescent RNAP Assay
Guardian Peroxidase Conjugate Stabilizor Diluent	Pierce	Western Blot
Hepes	Sigma Aldrich	Buffer
Histidine Tag	Dianova GmbH	Western Blot
Imidazol	Sigma Aldrich	Buffer
Immuno Pure Antibody (Anti Goat- Mouse IgG labelled HRP)	Pierce	Western Blot

IPTG	Roth	Inducer
iso-Propanol	J. T. Baker	Various uses
JCSG+ Suite	Qiagen	Crystallization Trial
Kanamycin	Sigma Aldrich	Antibiotics
Mercaptoethanol	Roth	SDS
Methanol		Various Use
Middlebrook ADC	BBL	Mycobacterial Medium
MnCl	Sigma Aldrich	Buffer
N,N,N',N'-Tetramethylethylenediamin	Sigma Aldrich	SDS
NAD	Roth	Enzyme Assay
NADH	Sigma Aldrich	Enzyme Assay
NADP	Sigma Aldrich	Enzyme Assay
NADPH	Roth	Enzyme Assay
Ni-NTA	Qiagen	Protein Purification
OADC Encrichment	BD	Enrichment for Medium
PACT Suite	Qiagen	Crystallization Trial
PageRuler™ Prestained Protein Ladder	Fermentas	Protein Standard
PageRuler™ Protein Ladder	Fermentas	Protein Standard
Peroxidase Conjugate-Goat Anti-Mouse IGM	Sigma-Aldrich	Western Blot
Poly(ethylene glycol)	Sigma-Fluka	Various Use
Polyoxyethylene Sorbitan Omon oelate (Tween 80)	Sigma Aldrich	Medium
Potassium Chloride	Fluka	Various Use
Potassium dihydrogenphosphate	Merck	Various Use
Potassium Iodide	Serva	Buffer
Potassium Sulphate	Roth	Buffer
Q Sepharose	GE Health care	Protein Purification
QIAquick Gel Extraction Kit	Qiagen	PCR Extraction
RNaseA	Qiagen	Buffer for MiniPrep
SDS ultra	Roth	SDS
Sephadex	GE Health care	Protein Purification
Sodium Chloride	Roth	Various Uses
Sodium dihydrogen Phosphate	Sigma Aldrich	Various Uses
Sodium Hydroxide	Roth	Various Uses
TaKaRa LA Taq + Mg plus buffer	Mobitech	PCR Kit
Taq DNA Polymerase	Qiagen	PCR Kit
TMB/Substrate Solution	Seramum, Diagnostic GmbH	Western Blot
Tris-Base	Sigma Aldrich	Buffer
Tris-HCl	Sigma Aldrich	Buffer
Trypton	BD	Medium
Urea	J. T. Baker	Protein Denaturation
Yeast Extract	BD	Medium
Zinc Chloride	Sigma Aldrich	Buffer

2.1.2 For RNAP assay

Table 3. Chemicals used for RNAP assay

Chemicals	Company
Adenosine 5′-phosphosulfate sodium salt	Sigma Aldrich
Alkaline Phosphatase, Calf Intestinal	Fermentas
Apyrase	NEB
ATP Determination Kit	Biaffin GmbH
ATP Sulfurylase	NEB
CTP, 100mM Solution	Fermentas
GTP, 100mM Solution	Fermentas
Manganese Chloride tetrahydrate	Sigma Aldrich
Ribonucleic Acid Polymerase from *E. coli*	Sigma Aldrich
Sodium Pyrophosphate Decahydrate	Sigma Aldrich
UTP, 100mM Solution	Fermentas
Rifampicin	Sigma Aldrich
Corallopyronin	HZI
Sorangicin	HZI
Ripostatin	HZI

2.1.3 For Pyrazinamidase assay

Table 4. Chemicals used for PZase assay

Chemicals	Company
α-Ketoglutaric Acid	Sigma Aldrich
Ammonium Iron (II) Sulphate Hexahydrate	Sigma Aldrich
L-Glutamic Dehydrogenase	Sigma Aldrich
Nicotinamide	Fluka
Nicotinic Acid	Sigma Aldrich
Pyrazinamide	Sigma Aldrich
Pyrazoic Acid	Sigma Aldrich

2.1.4 Drug candidates from HZI (former GBF)

Table 5. List of CP like substances

Substances	Name/Order Name	Company
Substance 16	CP14a	HZI
Substance 17	CP-17	HZI
Substance 18	CP-18	HZI
Substance 19	CP-19	HZI
Substance 21	CP-21a	HZI
Substance 24	CP-24a	HZI
Substance 25	CP-25a	HZI
Substance 28	CP-28a	HZI
Substance 30	CP-30	HZI
Substance 55	GW 387	HZI
Substance 56	GW 386	HZI
Substance 57	GW 389	HZI
Substance 58	GW 341	HZI
Substance 59	GW 342	HZI
Substance 60	CP40	HZI
Substance 61	CP 43	HZI
Substance 62	CP 44	HZI
Substance 63	CP45	HZI
Substance 64	CP46	HZI
Substance 65	CP47	HZI
Substance 66	CP48	HZI
Substance 71	GW414	HZI
Substance 74	CP 57	HZI
Substance 75	CP 58	HZI
Substance 76	CP 59	HZI
Substance 77	CP 60	HZI
Substance 78	CP 61	HZI
Substance 79	CP 63	HZI
Substance 80	CP 64	HZI
Substance 81	CP-66	HZI
Substance 82	CP 68	HZI
Substance 83	CP 69	HZI
Substance 84	CP70	HZI
Substance 85	CP65	HZI
Substance 86	CP73	HZI
Substance 87	CP74	HZI
Substance 88	CP75	HZI
Substance 89	CP76	HZI

2.1.5 Oligonucleotides

Table 6. List of Oligonucleotides

Primers for *rpoA*

Name	5′-3′ Sequence	No.
rpoA-F	CGAGTGCCCCCACAGACGTCATAT	P581
rpoA-R	ATGTGAAGACGACCCGCCGA	P584
rpoA-HindIII-R	GCGCGAAGCTTCTAAAGCTGTTCGGTTT	P582
rpoA-NdeI-F	CTTATGACATATGCTGATCTCACAGCGCCC	P583
rpoA-NdeI-His-F	CTGCATGCATATGCATCATCACCACCACCATCTGATCTCACAG CGCCCCAC	P639
rpoA-HindIII-His-R	AGGCAGTAAGCTTCTAGTGGTGGTGGTGGTGATGAAGCTGTTC GGTTT	P640

Primers for *rpoB*

Name	5′-3′ Sequence	No.
rpoB-Fw1	GCATCTTGGCAGATTCCCGC	P436
rpoB-Rv1	GCTTTACGCAAGATCCTCGACA	P437
rpoB-NdeI-His-F	TTGGTCGCATATGCACCACCATCATCACCACTTGGCAGATTCC CGCCAGAGCAAA	P484
rpoB-NdeI-F	GGAAGGACATATGTTGGCAGATTCCCGCCAGAGCAAA	P485
rpoB-HindIII-R	ATTTTGCGTTCGAATTACGCAAGATCCTCGACACT	P486
rpoB-HindIII-His-R-	CCTAACAAGCTTTTAATGATGGTGATGGTGGTGCGCAAGATCC TCGACA	P488

Sequencing primers for *rpoB*

Name	5′-3′ Sequence	No.
rpoB+1074Fs	GGTGGAAACCGACGACAT	P451
rpoB+2072Fs	AGGCCGGTCAGGTGATCG	P452
rpoB+3067Fs	TACATCATGAAGCTGCAC	P453
rpoB+2912Rs	AACACCGGCGTCGACACAA	P454
rpoB+1921Rs	TTTCTTCGGCGACGACGA	P455
rpoB+925Rs	TATAGCGACCGACGCGGG	P456
rpoB+578Fs	TGCACAGCGTCAAGGTGA	P476
rpoB+1829Fs	TGCCGCTGGTCCGTAGCGAGG	P478
rpoB+2581Fs	GAGCTGGTGCGTGTGTAT	P479
rpoB+3392Rs	CACCGTCACTCGATAGCA	P480
rpoB+2407Rs	TCAGCTCGGTCTCACCCTT	P481
rpoB+1404Rs	TAGTGCGACGGGTGCACG	P482
rpoB+400Rs	ACCGGTGTTGTTGTTGAT	P483

Primers for *rpoC*

Name	5′-3′ Sequence	No.
rpoC-F2	AACTGCGCGAAGGTGAGGAC	P597
rpoC-R2	TTGCCACTCATGTTGAACGG	P595
rpoC-NdeI-F	TCCGTCGCATATGCTCGACGTCAACTTCTT	P589
rpoC-NdeI-His-F	TTGGTCGCATATGCACCACCATCATCACCACGTGCTCGAC GTCAACTTCTTCGAT	P764
rpoC-EcoRI-R	GTCAGTGAATTCCTAGCGGTAGTCGCTGTA	P587

Sequencing primers for *rpoC*

Name	5′-3′ Sequence	No.
rpoC-seqF-1	GTGCTCGACGTCAACTTC	P733
rpoC-seqF-401	ACGTGATCACCTCGGTCGA	P734
rpoC-seqF-802	TTCGACATCGACGCCGAA	P735
rpoC-seqF-1201	TCGCTTTCCGATCTGCTC	P736
rpoC-seqF-1604	ACTTCGACGGTGACCAGA	P737
rpoC-seqF-2007	CCGGGTGATGTTCAACGA	P738
rpoC-seqF-2401	ACCATCGTCGACTCCGGC	P739
rpoC-seqF-2803	AACGTCATCGTCGAGCGT	P740
rpoC-seqF-3213	CGGTGAGGAAGTGGTCTA	P741
rpoC-seqF-3617	TGCTGATGGGCATCACGA	P742
rpoC-seqR-3948	CTAGCGGTAGTCGCTGTA	P743
rpoC-seqR-3539	ATCAGCGAGCCAGGCAAA	P744
rpoC-seqR-3148	CGGTGACGTCGGCGATCG	P745
rpoC-seqR-2744	ATGTACGGGTCGCGGATCA	P746
rpoC-seqR-2345	TCGGTGGCTTCCTTCCAA	P747
rpoC-seqR-1948	ATAGCTCGGCCTCGATCT	P748
rpoC-seqR-1548	CACCAGCATTGGCTCGAA	P749
rpoC-seqR-1131	CACGGATTCCTGCAGCAT	P750
rpoC-seqR-734	TCGACGAGTTCGCGGTAGA	P751
rpoC-seqR-331	AGGGCACACCCTTGAAGT	P752

Primers for *rpoZ*

Name	5′-3′ Sequence	No.
rpoZ-F	CTAGTCAGTATTTAGCTTTCCA	P591
rpoZ-R2	ACTATTACCTGCTTGGGGAT	P596
rpoZ-NdeI-F	CTGCATGCATATGAGTATCTCGCAGTCCGA	P593
rpoZ-NdeI-His-F	CTGCATGCATATGCATCATCACCACCACCATAGTATCTCG CAGTCCG	P599
rpoZ-HindIII-R	AGACTTAAAGCTTCTACTCGCCCTCGGTGT	P592
rpoZ-HindIII-His-R	CGCCTATAAGCTTCTAATGGTGATGGTGATGATGCTCGCC CTCGGTGTG	P600

Primers for *pncA*

Name	5′-3′ Sequence	No.
pncA-F2	CGGCGTCATGGACCCTATA	P603
pncA-R2	GAACCCACCGGGTCTTCG	P604
pncA-NdeI-F	TGGTGGACATATGCGGGCGTTGA	P792
pncA-Xho-SR	GTTCGGCGCTCGAGTCAGGAGCTGCA	P793
pncA-Xho-SR	GGCGGTGCTCGAGGGAGCTGCAAAC	P794

Sequencing primers for *pncA*

Name	5′-3′ Sequence	No.
pnca-sF1	CATTGCGTCAGCCGTACT	P633
pncA-sF2	CCACCGATCATTGTGTGC	P634
pncA-sr1	GCAGCCAATTCAGCAGTG	P635
pncA-sr2	GGTCGATGTGGAAGTCCT	P636

Primers for *amiD*

Name	5′-3′ Sequence	No.
amid-F2	CGGCGGCGCTGAAGTATCTGTT	P785
amidR2	ACCTTCGGTGCGACATTCGC	P786
amiD-NdeI-F	TGATTAGCCATATGACCGATGC	P790
amiD-HindIII-R	CGGCCGAGAAGCTTTCACACCGGCGG	P791

Sequencing primers for *amiD*

Name	5′-3′ Sequence	No.
amiD727F	ACGCTGCGGCGTATCGAAA	P777
amiD1130F	GATTGTGCTTCGGCTCGA	P778
amiD1514F	TCGACGTCAAGCTGCCCG	P779
amiD1930F	CAGTTCATCGGCCGTGAA	P780
amiD1722R	CGATCGCGTGAATTCCAG	P781
amiD1323R	GACACTGAGCAATACCGC	P782
amiD907R	TCGGGGCGTCGACCGTGTA	P783
amiD521R	TTCGACACCGAACCGGAT	P784

Other sequencing primers

Name	5′-3′ Sequence	No.
T7-promoter	TAATACGACTCACTATAG	P404
T7-Terminator	GCTAGTTATTGCTCAGCGG	P406
Rv2044cF	TGGCGAATTGAACCTTTTCT	P771
Rv2044cR	CACCCTCGCAGAAGTCGT	P772
TranspSF	ACCGAAGAATCCGCTGAG	P773
TranspSR	TCGCGTCGAGGACCATGGA	P774
pLEXSR-seq-F	GGTGACCCCCGTTTCATC	P637
pLEXSR-seq-R	GATGCCTGGCAGTCGATC	P638

2.2 Instrument used

Table 7. Instruments used

Instrument	Company	Purpose
Dark Reader Transilluminator	Clare Chemical.com	Blue Light to visualize DNA
Dynex Microplate reader MRX Revelation	Dynex	96 well plate reader
MRXTC Revelation	Dynex	96 Well plate reader
POLARstar OPTIMA	BMG Labtech	Luminescence Measurement
Sigma Blotter Semi-dry	Sigma Chemical Co.	Blotter
Sorvall RC-5B Refrigerated Superspeed Centrifuge	Sorvall	Centrifuge
Ultrasonicator	Braun Biotech International GmbH	Ultrasonication
Fluor-S MultiImager	Bio-Rad	Agarose Gel Photography
Millipore Multi Screen HTS-Vacuum Manifold	Millipore	Vacuum Accessory
Vacuum Controller PVK 610 (Vacu Box)	MLT AG	Vacuum Pump

2.3 Software used

Table 8. Softwares used

Software and version	Company	Usage
Vector NTI Advance 10 ver 10.1.1	Invitrogen Corporation	Primer Design & Molecular Analysis
Staden Package Gap ver 4.10 Pregap		DNA sequence Analysis - DNA Alignment, Editing, Mutation Analysis
FinchTV v1.4.0	Geospiza, Inc	DNA Sequence Reading
CellDesigner	Systems Biology Institute	Molecular Modelling
SP 20000 ver 6.10.6.2	SAFAS monaco	Reaction Kinetics Measurement
Prism v 5.01	GraphPad	Enzyme Characterization
Bioworks package v 3.5	Beckmann Instruments, Inc	Biomek 2000 Roboter
MDL ISIS/Draw 2.5	MDL Information Systems, Inc	Drawing Chemical Reactions

2.4 Bacterial strains and culture procedures

2.4.1 Bacterial strains

Table 9. *E. coli* strains used in this study.

E. coli strain	Genotype and relevant phenotype	Reference
E. coli Tuner (DE3)	F- *ompT hsdSB* (*rB - mB* -) *gal dcm lacY1* (DE3)	Novagen
E. coli BL21 (DE3)	F- *ompT hsdSB* (*rB- mB* -) *gal dcm* (DE3)	Novagen
E. coli Nova Blue	*endA1 hsdR17(rK12 - mK12$^+$) supE44,thi-1 recA1, gyrA96 relA1 lac*[F´*proA$^+$B$^+$, lacI^{q}ZΔM15*::Tn*10*(Tc^R)]	Novagen
E. coli DH5α	*supE44* Δ*lac*U169(Φ80 *lacZ* ΔM15), *hsdR17 recA1 endA1 gyrA96 thi*-1 *rel*A1	(Hanahan 1983)
E. coli Top 10F´	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*), 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen

All *Mycobacterium* strains used in this study are listed in

Table 10. Cell lysates and DNA from *M. tuberculosis* were kindly provided by Dr. W. Oehlmann (Lionex GmbH, Braunschweig, Germany).

Table 10. Mycobacterial strains used in this study

Mycobacterial Strain	Code	Origin
M. bovis BCG Chicago	ATCC 27289	DSMZ, Braunschweig, Germany.
M. bovis BCG Copenhagen	Danish strain 1331	Statens Serum Institut, Copenhagen, Denmark.
M. marinum; DSMZ 44345	ATCC 11564	DSMZ, Braunschweig, Germany.
M.smegmatis mc^2 155	ATCC 700084	DSMZ, Braunschweig, Germany.
M. tuberculosis H37Rv	ATCC 25618	C. Espitia, Mexico City, Mexico.

2.4.2 Cultivation of *E. coli*

E. coli DH5α and *E. coli* T*op 10 F´* were used for transformation and propagation as well. The strain *E. coli* BL21 (DE3) was used for the expression of recombinant protein under the control of the T7 RNA polymerase promoter. The *E. coli* Tuner (DE3) and *E. coli* Nova Blue were also used for expression of RpoB. The *E. coli* cells were cultured at 37 °C in LB medium on a rotary shaker operated at 160 rpm (INFORS AG), in

Erlenmeyer flasks filled up with not more than 20 % of their total volume. For strains with plasmids, selection was done by supplementing the medium with appropriate antibiotics either 100 µg/ml ampicillin or 50 µg/ml kanamycin.

Table 11. Composition of LB Medium

Tryptone (BD 211705)	10.0 g
Yeast Extract (BD 212750)	5.0 g
NaCl	10.0 g
Distilled water	up to 1 L
Adjust to pH 7.4 with NaOH (per 1 L 1 ml 1M NaOH)	

Alternatively for the purpose of auto induction of protein, APS Agar medium with appropriate antibiotics was used.

Table 12. Composition of APS medium

Difco Select APS Super Broth	49.1 g
Glyerol 87%	5 ml
Deionized Water	fill upto 1 L
Check pH	pH 6.8-7.5
Agar	15 g

Approximate Formula* per liter

Soy Hydrolysate	12.0 g
Yeast Extract	24.0 g
Dipotassium Phosphate	11.4 g
Monopotassium Phosphate	1.7 g
*****Adjusted and/or supplemented as required to meet performance criteria.	

2.4.3 Cultivation of Mycobacteria

Culture conditions for *M. bovis* BCG, *M. marinum* ATCC11564 and *M. smegmatis* mc^2 155 were 37 °C in Middlebrook 7H9 broth (BD) with ADC enrichment (BD) or on Middlebrook 7H10 agar (BD) with OADC enrichment (BD). Liquid culture was done with moderate shaking of 160 rpm. For recombinant *M. smegmatis* mc^2 155 and *M.*

bovis BCG, selection was performed by addition of 50 μg/ml kanamycin to the medium. Alternatively *M. smegmatis* mc^2 155 was cultured in LB medium or LB Agar.

Table 13. Composition of Middlebrook 7H9 broth

Middlebrook 7H9 medium (1 L)	
Bacto-Middlebrook 7H9 Broth	4.7 g
Glycerol (87 % (w/v))	2 ml
Water up to	900 ml
Autoclave and add 100 ml ADC Enrichment	

Table 14. Composition of Middlebrook 7H10 agar

Middlebrook 7H10 agar (1 L)	
Bacto-Middlebrook 7H10 Agar Base	19 g
Glycerol (87 % (w/v))	5 ml
Water up to	900 ml
Autoclave and add 100 ml OADC Enrichment	

2.4.4 Master cell bank

Strains grown and selected in LB plates with antibiotics were used for temporary storage. The plates with clones can be used for a month when stored at 4°C. Glycerol stock was prepared for clones for a long term storage and stored at –20°C or –80°C. A master cell bank was made from the clones and preserved at –80°C for indefinite storage.

2.5 Plasmids used

Table 15. Plasmids used in this study

Plasmids	Properties	Reference
pUC18	Cloning vector (2.7 kb) containing portion of pBR322 and M13mp19, *lacZα*, pMB1 origin, *bla*, ampicillin resistant	Fermentas (GeneBank accession number L09136)
pET-22b(+)	IPTG-inducible expression vector (5.5 kb), T7 promoter, T7 terminator, C-terminal His-tag, lac operator, pBR322 ori, ampicillin resistant	Novagen
pET-26b(+)	IPTG-inducible expression vector (5.4 kb), T7 promoter, T7 terminator, N-terminal His-tag, lac operator, pBR322 ori, *pelB* coding sequence, kanamycin resistant	Novagen
pET-28b(+)	IPTG-inducible expression vector (5.4 kb), T7 promoter, T7 terminator, N-terminal His-tag with thrombin restriction site, lac operator, pBR322 ori, kanamycin resistant	Novagen
plexSR5	Modified pMV261 with *hsp65* and kanamycin resistant.	Lionex GmbH
plexSR6	As plexSR5 except it contains additionally GCAATGGCCAAG after *hsp65*.	" "
plexSR9	Modified pMV261 with *hsp65*, His and thrombin tagged and kanamycin resistant.	" "

2.6 Methods of gene manipulation and analysis

2.6.1 Isolation of plasmid

2.6.1.1 Miniprep

Miniprep was done for the isolation of up to 20 µg of plasmid from 2-4 ml of culture. The isolation of plasmid was done with QIAprep Miniprep (Qiagen) and according to the manufacturer's instruction.

Briefly, a colony of candidate clone was incubated in 5 ml or 20 ml LB media overnight with appropriate antibiotic. 3 or 4 ml of the culture was pelleted in 2 ml microcentrifuge tube. The pelleted bacterial cells were resuspended in 250 µl of Buffer P1 (Resuspension buffer) then lysed with 250 µl of P2 Buffer (Lysis buffer) containing NaOH by gently inverting the tube for 4-6 times. 300 µl of Buffer N3 (Neutralization buffer) was added to neutralize the effect of NaOH in lysis buffer and mixed gently by

inverting the tubes for several 4-6 times, followed by centrifugation at 13,000 rpm for 10 min. The supernatant obtained was pipetted in QIAprep spin column supplied by the manufacture and centrifuged for 1 min to bind the plasmid on the membrane of the spin column. After flow-through was discarded, the spin column was washed with 0.5 ml Buffer PB (Equilibration buffer) and centrifuged and the flow-through was discarded. The column was washed again with 0.75 ml Buffer PE (Wash buffer) and again centrifuged. After the flow-through was discarded, the column was centrifuged once again to remove any residual buffer left, then the spin column was placed inside a clean microcentrifuge tube and 50 µl of elution buffer (10mM Tris-HCl, pH 8.5) was added at the centre of the membrane waited for a minute. The elution was done with centrifugal force generated with 1 min centrifugation.

Mini-scale isolation of plasmid DNA was used for the preparation of recombinant plasmid for sequencing and retransforming. Qiagen miniprep was used according to the manufactures instruction for the isolation of the recombinant plasmid.

Table 16. Composition of Buffer P1 (Resuspension buffer)

Tris-Base	6.06 g
$Na_2EDTA.2H_20$	3.72 g
Water	Up to 1 L
pH adjusted to pH 8.0	
RNase A	100 mg

Table 17. Composition of Buffer P2 (Lysis buffer)

NaOH	8.0 g
Water	Up to 950ml
20% SDS (w/v)	50ml

Table 18. Composition of Buffer N3 (Neutralization buffer)

Potassium Acetate	294.5 g
pH adjusted to 5.0 with glacial acetic acid	
Water	Up to 1000 ml

2.6.1.2 Maxi Prep

When a larger quantity of plasmid was required e.g. for RNAP Assay, Maxi prep was done according to manufacturer's instruction. With maxi prep up to 500 µg of plasmid can be gained at once.

A colony of *E. coli* Top 10F´ with pUC18 was cultivated in 100 ml LB medium supplemented with 100 µg/ml ampicillin. The cell pellet was harvested at 6000 x g for 15 min at 4°C. The pellet was homogenously resuspended in 10 ml Buffer P1 (Resuspension buffer), then mixed thoroughly with 10 ml Buffer P2 (Lysis buffer) by vigorously inverting 4-6 times and incubated at room temperature for 5 min to lyse the cells. During the incubation time, QIAfilter cartridge was prepared by screwing the cap onto the outlet nozzle of the QIAfilter Maxi cartridge. After the incubation time was over, 19 ml of chilled Buffer P3 (Neutralization buffer) was pipetted and mixed thoroughly by vigorously inverting 4-6 times to neutralize the effect of lysis. The bacterial lysate was cleared by pouring the lysate into the barrel of the QIAfilter cartridge and incubating at room temperature for 10 min. The cap from the QIAfilter cartridge outlet nozzle was removed and gently inserted into the QIAfilter Maxi cartridge and the cell lysate was filtered into a 50 ml tube. 2.5 ml of Buffer ER as added to filtered lysate, mixed by inverting the tube for 10 times and incubated on ice for 30 min. Qiagen-tip 500 was equilibrated by applying 10 ml of Buffer QBT and allowing the column to empty by gravity flow. The filtered lysate was applied to the QIAGEN-tip to bind plasmid to the resin. The QIAGEN-tip was washed with 30 ml of Buffer QC (Wash buffer) to remove unnecessary contaminants. Elution of bound DNA was done with 15 ml of Buffer QF (Elution buffer). The eluted DNA was precipitated by adding 10.5 ml of isopropanol. The precipitated DNA was pelleted by centrifuging it at 15,000 x g for 30 min at 4°C. Supernatant was decanted and the DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 15,000 x g for 10 min. Once again the supernatant was decanted carefully. The pellet was air-dried and the DNA was redissolved in 500 µl of Buffer TE (10mM Tris-HCl, 1mM EDTA pH 8.0).

2.6.1.3 DNA preparation in 96 well plate

When a large number of plasmids are to be isolated, 96 well plate DNA preparation was preferred. Bacterial cells were cultivated in 1.6 ml LB medium with 50 µg/ml kanamycin at 37°C in a 96 deep well plate covered with air permeable foil in 230 rpm rotating shaker (INFORS AG). The overnight grown cells were centrifuged at 1500 x g for 5 minutes to pellet the cells. The supernatant from the deep well was carefully decanted. The cell pellets were then homogenously resuspended in 80 µl of suspension buffer by vortexing vigorously. The cell suspension was then mixed with 80 µl of denaturation buffer. 80 µl of neutralization buffer was pipetted and shaken in microtiter plate shaker (IKA Werke GmbH & Co. KG) for a couple of minutes. The lysate was transferred in 96 well Multiscreen HTS Nucleic A or NA plate (Millipore) and suction was applied with Vacuum Controller PVK 610 (MLT AG) with another 96 well Multiscreen HTS, FB or FB plate (Millipore) below the NA-plate in Millipore Multi Screen HTS-Vacuum Manifold (Millipore). The lysate passed from the NA-plate to FB plate during the suction. 150 µl of binding solution was pipetted in FB plate to bind the DNA. Washing was done with 200 µl of 80% Ethanol twice. The FB plate was dried at 65°C for an hour. Elution was done with 35 µl water twice by applying the vacuum suction with another 96 well plate below FB plate. The first elution was done by wetting the FB plate at 37°C for a minute.

Table 19. Composition of Suspension buffer (30 mM Glucose, 15 mM Tris-HCl pH 8.0, 30 mM Na_2EDTA, 60 µg/ml Rnase A)

1 M Glucose (Sterile Filtered)	6 ml
0.3 M Na_2EDTA	12 ml
1 M Tris-HCl pH 8.0	3 ml
RNase A (10 mg/ml)	12 µl
MilliQ Water	179 ml
Storage at 4°C.	

Table 20. Composition of Denaturation buffer (500 ml) Storage at RT

5 M Sodium Hydroxide	20 ml
10% SDS	50 ml
MilliQ Water	430 ml

Table 21. Composition of Neutralization buffer (500 ml) Storage at RT

Conc. Acetic Acid	70 ml
5 M Potassium acetate	360 ml
MilliQ Water	70 ml

Table 22. Composition of Binding solution (6.1 M Potassium Iodide)

Potassium Iodide	280 g
MilliQ Water	196 ml

Dissolve overnight, Store at RT protect from light.

2.6.2 Polymerase chain reaction (PCR)

PCR was performed in different volumes, 10 µl-100 µl according to the necessity. 10 µl was used for the optimization of a PCR e.g. during gradient PCR. For sequencing purpose 20 µl of PCR reaction was sufficient. For cloning purpose 50-100 µl of PCR reaction was used. For all individual PCR reactions, initial optimization of PCR was performed with gradient PCR.

Table 23. Reaction composition using Taq DNA polymerase (Qiagen/Biomix)

Component	Volume (µl)	Final Concentration
Taq PCR Master Mix	50	2.5 U
Forward Primer	1	0.1 µM
Reverse Primer	1	0.1 µM
Distilled Water		
Template DNA		≤ 1 µg/Reaction

Table 24. Reaction mixture for PCR using TaKaRa enzyme (total 100µl)

Component	Volume (µl)	Final Concentration
TaKaRa LA Taq ™ (5 U/µl)	1	2.5 U
10x Buffer II	10	
Forward Primer	1	0.1 µM
Reverse Primer	1	0.1 µM
Template DNA		≤ 1 µg/Reaction
Distilled Water	Up to 100	
Total	100 µl	

Table 25. PCR reaction condition

Denaturation	95°C	10 min	
Denaturation	95°C	30 sec	
Annealing	52 °C	30 sec	30x
Extension	72°C	X min*	
Final Extension	72°C	10 min	
Storage	4°C	forever	

* Extension time depends upon the length of PCR product. For PCR products longer than 1kb, extension time of approximately 1 min per kb DNA was calculated.

2.6.3 Agarose gel electrophoresis of DNA

The size of DNA fragments or entire plasmids was determined by analysis on 0.8%, 1% or 1.5 % agarose gels. The lower the size of DNA the higher the percentage of gel used. The necessary amount of agarose was dissolved in 1x TAE buffer. DNA was loaded with an appropriate dilution in a 2 or 6-fold loading buffer (Fermentas) onto the gel using 1 x TAE as running buffer. The electrophoresis was performed at 80 - 100 Volts with Horizon 58 gel Chamber (Bethesda Research Laboratories) or DNA Sub Cell gel chamber (Bio-Rad) for 30-45 minutes. The agarose gel was stained in a GelStar Nucleic Acid Stain (Lonza Verviers) in 1x TAE for 10 min. Photography of the agarose gel was done under UV light with Fluor-S MultiImager (Bio-Rad). Alternatively for visualization and successive excision of DNA required for the extraction of DNA band from agarose gel, Dark Reader transilluminator (Clare Research) was used.

Table 26. Composition of 50 x TAE buffer:

Tris-Base	242 g
Glacial Acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Water up to	1000 ml

2.6.4 DNA extraction

2.6.4.1 With Qiagen Kit

For small numbers of DNA, Qiagen Gel Extraction kit was used for the extraction of DNA of size less than 10 Kb. The kit can recover up to 10 µg of DNA. Plasmids restricted with restriction enzyme were also extracted with this kit. The kit was also used both for the extraction of PCR products or PCR products from the agarose gel.

The DNA fragment was excised from agarose gel with a clean scalpel under blue light using Dark Reader transilluminator (Clare Research). The excised DNA fragment was weighed and 3 volume of Buffer QG was added to dissolve the agarose by incubating at 50°C for 10 min in shaking Thermomixer 5436 (Eppendorf). After the agarose was dissolved 1 gel volume of isopropanol was added in the sample and mixed. The sample was applied in QIAquick column and centrifuged for a minute. The flow-through was discarded and the QIAquick column was washed again with 0.5 ml of Buffer QG. After 1 minute centrifugation the flow-through was discarded. Final washing was done with 0.75 ml of Buffer PE and after 1 minute centrifugation flow-through was discarded. Residual ethanol left in the column was removed by 1 min centrifugation. Elution was done in 1.5 ml microcentrifugation tube with 30 or 50 µl MilliQ water.

2.6.4.2 PEG precipitation

PEG precipitation method was used when DNA extraction from large number of samples was necessary. 20 µl of 10% PEG 8000 was mixed with 20 µl of PCR product in 96 well plate and mixed properly by vortexing. The plate with the mixture was incubated for 10 min at RT and then centrifuged at 3000 x g for 30 min at 4°C. The supernatant was carefully decanted by turning the plate upside down and gently tapping it. Complete removal of the supernatant was achieved by centrifuging the plate for a minute at 100 x g with the wells facing downwards. Washing of the pellet was done with 100 µl of 80% ethanol. The ethanol was decanted and the residual ethanol was

removed by centrifuging the plate for a minute at 100 x g with the wells facing downwards. The plate was air-dried and the pelleted DNA was solubilized in 10 µl of MilliQ water.

2.6.5 Quantitation of nucleic acids

2.6.5.1 Measurement of absorbance

The qualitative and quantitative analysis of DNA was done with a UV spectrophotometer using a quartz cuvette. A nucleic acid solution containing 50 µg/ml of ds DNA has absorbance (Optical Density) of 1.0 at wavelength 260 nm. The concentration dependent absorbance of DNA at 260 nm was used to estimate the amount of DNA in a solution. DNA was diluted 1:10 or 1:100 with water. 100 µl of the solution was added into a quartz cuvette and absorbance was measured at 260 nm and 280 nm against water as reference.

Pure preparations of DNA and RNA have OD_{260}/OD_{280} of 1.8 and 2.0 respectively. Qualitative analysis of DNA was controlled by calculating ratio of OD at 260 nm and OD at 280 nm if the solution contains pure DNA. If the measured ratio is smaller, it indicates a contamination.

2.6.5.2 Quantitation in agarose gels

Alternatively to the method described above, the concentration of nucleic acids was be approximated by staining the DNA in agarose gel with GelStar Nucleic Acid Stain (Lonza Verviers). A defined amount of GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) was used as a standard to run a gel in parallel with the DNA in question. The gel was documented with a Fluor-S MultiImager (Bio-Rad) and analyzed.

2.6.6 DNA sequencing

Automated non-radioactive sequencing of DNA was carried out according to the di-deoxy-method of Sanger (Sanger et al. 1977). The ABI PRISM Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used on a 3730xl DNA Analyzer (Applied Biosystems) according to the manufacturer's instruction for DNA sequencing.

2.6.7 DNA digestion with restriction endonucleases

In each restriction reaction an amount of approximately 3 units enzyme (Fermentas) was used for each microgram of DNA. The amount of buffer (ionic strength) depended on the specific application, sought from the company's website (http://www.fermentas.com). Restriction was done at 37°C for 2-4 hours. The inactivation of the enzyme depend upon the type of specific enzyme used.

2.6.8 Ligation

The molar ratio of insert to plasmid was approximately 3:1 was used for ligation. The reaction was carried out in a total volume of 20 µl in T4 DNA ligase buffer containing 1 unit T4 DNA ligase (Invitrogen or Fermentas) per 1 µg DNA. The reaction mixture was incubated at room temperature overnight.

2.6.9 Transformation of DNA into bacteria

2.6.9.1 Electroporation in *E. coli*

Electrocompetent *E. coli* cells were prepared as follows:

A single colony of *E. coli* was incubated overnight at 37°C at 160 rpm in LB broth to make a starter culture. From this starter culture 5 ml of the innoculum was inoculated in 1 L of LB broth containing low salt concentration (10 gm tryptone, 5 gm yeast extract, 5 gm NaCI per litre) and cultivated at 37°C with 160 rpm shaking. The cells were grown till the OD_{600} reached 0.5-0.9 (3-4 hours). The cells were then chilled on ice for 30 min and pelleted at 4000 x g at 4°C for 15 min. The pellet was resuspended and centrifuged for four successive washings. In the first washing 1000 ml of sterile cold water was used, in the second and the third washing 500 ml of sterile cold water was used and in the final washing 20 ml of sterile cold water was used. Finally dilution was done by resuspending the pellet in 2 ml of 10% sterile cold glycerol. An aliquot of 40 µl of the electrocompetent cells were frozen at –80°C for future uses.

For electroporation, 40 µl of the electrocompetent cells from –80°C were thawed in ice for 15 min. 1 µl plasmid (1 ng/µl in water) was mixed in chilled cuvette (*E. coli* Pulser cuvette, 0.2 cm gap; Bio-Rad). The cuvette was lightly tapped to mix and to settle down the cell mixture. Electroporation was done at 2.5 kV, 200 W and 25 µF with pulse time 3-5 ms. The cells were incubated at 37°C for 30 min in SOC medium with moderate shaking and plated on LB Agar with appropriate antibiotics.

Table 27. Composition of SOC Medium (SOB medium with 20 mM glucose)

SOB Medium	1 L
1 M glucose	20 ml

Table 28. Composition of SOB medium

Bacto tryptone	10 g
NaCl	0.5 g
KCl	0.186 g
Distilled water	up to 1 L
	10 mM $MgCl_2$*
*Add 10 ml/L 1 M sterile $MgCl_2$ just before use.	

2.6.9.2 Electroporation in *M. smegmatis*

For electroporation of *M. smegmatis* mc^2 155, 100 µl of the electrocompetent cells was used. Electroporation was carried out in room temperature. 10 µl plasmid (1 ng/µl in water) was mixed in precooled cuvette (*E. coli* Pulser cuvette, 0.2 cm gap; Bio-Rad). The cuvette was flicked to mix and settle cell mixture. Electroporation was done at 1.25 kV, 800 Ohm and 25 µF for 9 ms as suggested by the Bio-Rad (Bio-Rad). The cells were incubated at 37°C for 2 hour with moderate shaking and plated on LB Agar with kanamycin.

2.6.9.3 Electroporation in *M. bovis* BCG Copenhagen

For electroporation of *M. bovis* BCG Copenhagen 200 µl of the electrocompetent cells were thawed in ice for 15 min. 10 µl plasmid (1 ng/µl in water) was mixed in precooled cuvette (*E. coli* Pulser cuvette, 0.2 cm gap; Bio-Rad). The cuvette was flicked to mix and settle cell mixture. Electroporation was done at 1.25 kV, 600 Ohm and 25 µF for 9 ms (Bio-Rad). The cells were incubated overnight with moderate shaking and plated on Middlebrook 7H10 Agar with kanamycin.

2.6.9.4 Heat shock transformation

Chemicompetent cells were prepared as follows:

The method for preparing competent cells was a modification of the calcium chloride procedure. A colony of *E. coli* was picked and starter culture was grown overnight in LB medium. The strain was further cultivated in LB medium with initial OD_{600} of 0.05. The cells were grown at 37°C with moderate shaking at 160 rpm until the OD_{600} reached between 0.6 to 0.8 (log phase). 40 ml of the cells were pelleted by centrifugation at 3000 x g and resuspended in 20 ml cold 100 mM $CaCl_2$ followed by incubation on ice for 20 min. The cells were recentrifuged and resuspended in 4 ml cold 100 mM $CaCl_2$ twice and incubated on ice for 1 hour. 200 µl of the bacterial suspension was aliquoted into pre-chilled tubes. The aliquots were either stored at –80°C for future use or used for heat shock transformation.

A maximum of 3 µl of ligation reaction mix or 5 ng of pure plasmid was mixed gently with the competent cells for transformation. Incubation was done in ice for 30 min. Heat shock was done at 42°C for 45 sec followed by incubation of the cell in ice for 2 minutes. 800 µl of SOC Medium was added to the tube to rejuvenate the heat stressed cells. The cells were incubated at 37°C for 30 min with moderate shaking. The cells after pelleting were resuspended in 100 µl SOC Medium and plated in LB agar with appropriate antibiotics for selection of antibiotics resistant clones.

2.6.10 Selection of positive clones

2.6.10.1 Colony PCR

Colony PCR was done for the screening of the positive clones that grown in the LB plates supplemented with antibiotics in order to avoid the false negative clones. The same PCR condition described as before was used in order to amplify the inserted gene

from the recombinant *E. coli* except instead of DNA template colony of transformant was used.

2.6.10.2 Auto induction

A colony from transformants was streaked in LB plate with antibiotics and a replica in APS plate with antibiotics with a sterile toothpick. The LB plate was grown at different temperatures overnight. Protein expressed from *E. coli* grown APS plates were analysed in SDS-PAGE and checked for the over-expression of protein. The positive clones were the one which over-express protein of specific size.

2.7 Analysis of protein

2.7.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gel was done to check protein expression of clones or to check the purity of protein by separating the denatured proteins in electrical field. SDS-PAGE was performed with Minigel System (11 cm x 7 cm x 0.1 cm) (Biometra) as described by Laemmli (Laemmli 1970). The protein sample was diluted 1:2 in 2X loading buffer denatured at 95°C for 10 min, cooled down and 1/10 volume of 1 M DTT was added to disrupt the disulphide bonding in the proteins. PageRuler™ Protein Ladder (Fermentas) was run parallel to the protein samples for comparison of molecular mass of the separated proteins. Electrophoresis was performed at 60 V till the proteins accumulate in the stacking gel. Later a potential difference of 120 V was applied for separating the proteins in the running gel.

Table 29. Composition of Stacking gels (4.0% gel, 0.125 M Tris, pH 6.8)

	For 4 gels (ml)
Distilled Water	6.15
0.5 M Tris-HCI, pH 6.8	2.5
20% (w/v) SDS	0.05
Acrylamide/Bis-Acrylamide (30%/0.8% w/v)	1.34
10% Ammonium Persulfate	0.05
TEMED	0.01
Total Monomer	10

Table 30. Composition of 10% Separating gel in 0.375 M Tris-HCl, pH 8.8

	For 4 gels (ml)
Distilled Water	12.3
1.5 M Tris-HCI, pH 8.8	7.5
20% (w/v) SDS	0.15
Acrylamide/Bis-Acrylamide (30%/0.8% w/v)	9.9
10% Ammonium Persulfate	0.15
TEMED	0.015
Total Monomer	30

Table 31. Compositon of 15% Separating gel in 0.375 M Tris-HCl, pH 8.8

	For 4 Gels (ml)
Distilled Water	7.2
1.5 M Tris-HCI, pH 8.8	7.5
20% (w/v) SDS	0.15
Acrylamide/Bis-Acrylamide (30%/0.8% w/v)	15
10% Ammonium Persulfate	0.15
TEMED	0.015
Total Monomer	30

2.7.2 Staining methods

After running the gel, the gel was stained in coomassie blue solution containing 0.1% coomassie blue, 10% (v/v) acetic acid and 40% (v/v) ethanol and 50% de-ionized water. The gel in the stain was microwaved for 1-2 minutes until it just started to boil and the stain was fixed for 15 minutes by shaking. After fixing the stain, the gel was destained with 10% ethanol to remove unnecessary background in the gel. The gels were heated in microwave to achieve fast destaining process.

2.7.3 Western blotting

This is an analytical method for immunological detecting a specific protein. His-tagged protein was detected with this method. SDS-PAGE was used to separate denatured proteins. The separated proteins were then transferred to Immobilon polyvinylidene fluoride (PVDF) microporous membrane (Millipore) to bind proteins.

PDVF membrane was wetted in methanol and equilibrated in transfer buffer for 30 min. The SDS gel was also equilibrated in the same buffer. The transferring of the proteins from SDS gel to PDVF membrane was done at 15 V for 20-45 min in semi-dry Sigma Blotter. After the proteins were transferred into the membrane, the membrane was incubated in blocking solution (1% BSA in 1xTBS, Tween 0.005%) in order to minimize any unspecific antibody binding to the membrane. The membrane was washed thrice for 5 min each in TBS- Tween (1xTBS, Tween 0.005%) and then incubated overnight with a dilution of 1:1000 – 1:5000 of the Anti-his Antibody in TBS-Tween. The membrane was again washed as described before and incubated for one hour with an horseradish peroxidase conjugated anti-goat Mouse IgG (Pierce) diluted 1:30,000 in TBS-Tween. The washing procedure was repeated before incubating the membrane with the substrate 3,3´,5,5´-tetramethylbenzidine (TMB) (Seramum Diagnostic GmbH) for HRP until a blue signal was developed. Reaction was stopped by rinsing the blot with water.

Solutions used for Western blot

Table 32. 10 X Transfer buffer (800 ml)

Tris-Base	58 g
Glycin	29 g
SDS	3.7 g
Water	800 ml
	pH 8.3

Table 33. 10 X TBS (1 L)

NaCl	80 g
KCl	2.0 g
Tris-Base	61.0 g
Water	1000 ml
	pH 7.4

2.7.4 Determination of protein concentration

Protein concentration was determined by the method of Bradford (Bradford 1976) using the Coomassie Plus™ Protein Assay Reagent (Pierce) against bovine serum albumin (BSA) as a standard. 5 µl of protein sample was mixed with 100 µl Protein Assay Reagent then incubated at room temperature for 5 minutes in 96 well plate microplate (Greiner). The absorbance was determined at OD_{595} with MRXTC Revelation (Dynex) using Revelation v4.25 (Dynex). The protein concentration of the samples was generated with Revelation v4.25 (Dynex) using a calibration curve with BSA as standard.

2.7.5 N-terminal amino acid sequencing

The protein of interest was separated by SDS-PAGE (2.7.1), transferred to an Immobilon PVDF-membrane (Millipore) and stained in a freshly prepared cold coomassie solution for a minute. The bands could be seen after destaining with 20% ethanol. The band of interest was cut with a clean sharp scalpel, washed in water and given for sequencing. The N-terminal amino acid protein sequencing was performed with the protein-sequencer 494 (Applied Biosystems).

2.7.6 Gene expression

2.7.6.1 Chemical induction

Clone containing the gene of interest in pET vectors (Novagen) was grown in LB medium with specific antibiotics as a starter culture. The next day inoculation was done in 800 ml LB medium with 50 µg/ml kanamycin or 100 µg/ml ampicillin with the starting OD_{600} of 0.2. The cells were cultured for about 3 hours and checked for OD_{600}.

When the OD_{600} reached between 0.6-0.9 induction was performed with 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) and further incubated at 30°C for 3 hr or overnight according to the induced protein, empirically determined from previous low-scaled experiment. Harvesting of the cells was done by centrifuging the cell culture at 5000 x g for 10 min and the pellets stored at –20°C for further applications.

2.7.6.2 Auto induction

Auto induction in *E. coli* BL21 (DE3) was done according to the proprietary protocol from Lionex GmbH, Braunschweig, Germany. 3 ml of a suspension of starter culture cultivated in LB medium with appropriate antibiotics was spread in Qtray (Genetix) containing 200 ml APS medium with appropriate antibiotics to form a lawn culture, and the plates were incubated at different temperatures for 3-4 days. The bacterial layer was then scrapped with cell scrapper tipped with glass rod.

2.7.7 Preparation of crude cell extract

For work concerning protein, all procedures were carried out in cold condition or alternatively in ice. Cell pellet was suspended in buffer used for the equilibration of the column used for further purification at 5 ml per gram wet cell pellet mass. In case of cell pellet derived from APS medium, the pellets was first homogenized with Miccra D-9 (ART-moderne Labortechnik) for 3 x 1 minutes (250 V ~50Hz, 80W, 3.6A, A= 10,500 min-1) in appropriate volume of buffer because of smooth and sticky consistency of the cells, which do not readily allow the cells to suspend at ease. Cell lysis was performed by the mechanical ultrasonication (30 sec for 200 µl-1ml, alternatively 3 x 2.5 min for 20 ml). A maximum of 20 ml was sonicated at a time. Cell lysate was decanted from the cell debris after centrifugation at 18,000 rpm for 15 min.

Alternatively, when a large number of different cell extracts are required, chemical lysis with BugBuster Protein Extraction Reagent (Novagen) was done. It gently disrupts the cell wall of bacteria and liberates soluble protein without denaturing it. Streaks of cell

previously grown in APS medium for 3 days at 18°C was put in the wells of 96 well plate. The cells were suspended and subsequently lysis was done in 100 µl of BugBuster solution by shaking the plate in microtiter plate shaker (IKA) for 20 min.

2.7.8 Protein chromatography

For all chromatographic operations the FPLC systems BioLogic Workstation (Bio-Rad) and Pharmacia LKB system (Pharmacia) were used. All buffers were filtered through 0.2 µm membrane filter (Sartorius), subsequently degassed under a negative pressure. In all the purification processes, the column was first equilibrated with at least two column volume of buffer and then only the sample was loaded. Washing of the column was done with two column volume where necessary as in the case of Ni-NTA and Q-Sepharose, where the protein was bound to the column material.

2.7.8.1 Immobilized-metal affinity chromatography (Ni-NTA)

The purification of 6 x His-tagged recombinant proteins were carried out with Ni-NTA Superflow resin (Qiagen). As per the manufacture, it has ability of absorbing 5-10 mg per ml of resin. Elution of the bound protein was done with buffer containing 500 mM Imidazole. Different strategies like linear gradient (5 ml Ni-NTA resin) or batch elution (50 ml Ni-NTA resin) were used for the elution of bound protein.

2.7.8.2 Ion exchange chromatography Q-Sepharose

15 ml Q-Sepharose (GE Healthcare) in XK16 column (Amersham Bioscience) was used for ion exchange chromatography. The column was equilibrated with 150 ml of 50 mM Tris-HCl pH 7.8. Sample was loaded into the column, and then washed with 250 ml of

the same buffer. The proteins were eluted with a linear gradient of 0-100% of 50 mM Tris-HCl, 500 mM NaCl pH 7.8.

2.7.8.3 Desalting and buffer exchange

The buffer of protein solutions was changed by gel filtration using a 150 ml Sephadex G-25 (Amersham Bioscience) in XK50 column. For small amounts of protein, desalting was done in Hi Trap Desalting 5x5 ml column (Amersham Bioscience). 10 mM ammonium bicarbonate buffer was used for the buffer exchange.

Alternatively for buffer exchange, dialysis was performed twice. The first dialysis was done for 4 hours and the second dialysis overnight in a desired buffer. The molecular weight cut off of the membrane used depends upon the size of protein.

2.7.8.4 Protein concentration

For the concentration of small amount of protein Vivaspin 6, Vivaspin 20 and Vivacell 70 (Sartorius) were used. The appropriate molecular weight cut off was chosen specifically according to protein size for each protein. Alternatively, for a larger volume of protein concentration was done in PALL concentrator with stirrer was used. Pressure up to 2.5 bar with nitrogen was applied with stirring condition at 4°C till appropriate volume was reached.

2.7.8.5 Gel permeation with Sephadex G-25

For desalting and changing buffer of large quantity of protein, Sephadex G-25 (Amersham Biosciences) in XK50 column was used. While for small quantity HiTrap Desalting 5x5ml packed with Sepahdex G-25 Superfine column was used.

Sephadex is a bead-formed gel prepared by crosslinking dextran with epichlorohydrin. Gel filtration separates molecules according to their relative sizes. Sephadex G-25 has a fractionation range for globular proteins of 1000–5000 molecular weight. Large molecules are totally excluded while smaller sized molecules enter the beads to varying extents according to their sizes. Large molecules thus leave the column first followed by smaller molecules in the order of their decreasing size.

2.7.8.6 Gel filtration on a Superdex 200

Gel filtration with Superdex (Amersham Biosciences) provides the buffer exchange and separation of protein according to the size and thus the estimation of the protein size. A protein sample previously concentrated was applied in the volume of 2-5 ml in pre-equilibrated column. The proteins were separated according to their sizes.

2.8 Microbiological methods

2.8.1 Kirby-Bauer method for antimicrobial sensitivity testing

The Kirby-Bauer method known as the disk diffusion test was used for testing the efficacy of the test substances against *E. coli* DH5α. *E. coli* DH5α was grown to OD_{600} 0.2 and a 2 ml of the culture was evenly spread on the surface of solid LB agar in Qtray (Genetix) and wait for 5 minutes to dry the plate. UV-sterilized assay disc (Schleicher & Schuell) of Φ 6 mm were placed above the agar plate. 10 µl of test solution was impregnated with 100 µg/ml or 10 µg/ml concentration on the surface of the disc. The substance diffused through the media away from the disk creating a concentration gradient. The plates were incubated at 37°C overnight. A clear retardation of growth around the test substance disc represents the zone of inhibition and represents the sensitivity to the impregnated substance.

2.8.2 Resazurin microtiter assay

Resazurin microtiter assay (REMA) was carried out as described (Palomino et al. 2002)).

The assay was performed in 100 µl of Middlebrook 7H9 media supplemented with ADC enrichment (BD). A dilution of 1:3 was done in adjacent wells in the plate to yield different concentration of the test substance. Growth controls with solvent and sterility control without innoculum were also included in each plate. The innoculum was prepared from growing cultures of *M. marinum* ATCC 11564 from whic h turbidity was adjusted to 1/20 of McFarland. 100 µl of the innoculum was added to each 100 µl of the medium with test substance. The plate was covered, sealed in a plastic bag and incubated at room temperature in normal atmosphere. After 7 days growth of the organisms were measured in 96 plate reader then 30 µl of 0.01% sterile resazurin solution was added to each well, and the plate was reincubated overnight. A change in colour from blue to pink due to reduction of resazurin into resorufin indicated bacterial growth. MIC was defined as the lowest concentration of the substance that prevented this change in colour.

2.9 Biochemical methods

2.9.1 Determination of RNAP activity

2.9.1.1 Homogenous fluorescent assay

Initially for the establishment of RNAP Assay, fluorescent assay as described by Kozlov (Kozlov et al. 2005) was attempted. RNAP polymerase reaction was done in 10 µl mixture containing 20 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 1.5 mM $MnCl_2$, 0.1 mM EDTA, 25 µM GTP or Um-pppG (Guanosine-5´-triphosphate-γ-(4-methylumbelliferyl)-ester), 25 µM CTP, UTP, ATP or Um-pppA (Adenosine-5´-triphosphate-γ-(4-methylumbelliferyl)-ester), 1-5 µg of *E. coli* RNAP, 1 µg pUC18 plasmid DNA for 2 hr

at RT. After incubation, 2 µl of 0.5 M 3-[(N-1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxyp ropanesulfonic acid (AMPSO), pH 9.2, containing 0.1 U of alkaline phosphatase activity was added and incubation continued for 5 min followed by addition of 2 µl of 100 mM EDTA, pH 9.0. The samples were transferred to standard 96-well plates and the fluorescence was measured at 460 nm upon excitation at 355 nm. Reaction was also proceed by varying reagent volume, reagent concentration and reaction time.

2.9.1.2 New RNAP assay developed in this study

DNA dependent RNA polymerase (RNAP) synthesises RNA incorporating nucleotides, which in turn release pyrophosphate (PPi). A new combination of RNA polymerase followed by PPi quantitation by a non-radioactive method was developed. The RNAP reaction mixture contained 1 µl of *E. coli* K-12 RNAP (1.1 mg protein/ml, 500-1500 U/mg), 500 ng pUC18 plasmid DNA in 10 mM $MgCl_2$, 1.5 mM $MnCl_2$, 0.1 mM EDTA, 50 mM NaCl, 20 mM Hepes, pH 8.0 and 25 µM of each NTPs (ATP, UTP, GTP and CTP). The mixture was incubated for 30 minutes at 37°C for RNAP to synthesize RNA. Similar reaction performed by Kuhlman (Kuhlman et al. 2004) for 30 min resulted in at least 90% maximal RNA synthesis.

After 30 min of polymerization, 2 µl containing (50 mU) of apyrase was added in the reaction mix to degrade the residual NTPs present in the reaction mix, which may interfere in the final signal detection. The reaction was carried out for 10 min at 30°C. Deactivation of apyrase was done for 10 min at 85°C. Further adenosine-5´-phosphosulfate (5 µM) and ATP Sulfurylase (30 mU) was added in the reaction mix to synthesise ATP from PPi produced during the polymerization by RNAP. This step was carried out for 10 min at 30°C during which ATP sulfurylase converts PPi to ATP at the expense of adenosine 5´ phosphosulfate. A final denaturating of the enzymes present in the whole reaction was performed at 85°C for 10 min. Finally net ATP produced during the whole reaction was evaluated with ATP kit (Biaffin GmbH). ATP energizes the conversion of luciferin to oxyluciferin generating visible light, which is measured with POLARstar OPTIMA (BMG Labtech). The relative light unit detected from a reaction

mix correspond to the amount of ATP present in a particular reaction mix, which again corresponds to the amount of PPi produced during the polymerization reaction of RNAP.

Method for the evaluation of inhibitory effect of substances

The reaction mix containing 2 µl of *E. coli* RNAP in 10 mM $MgCl_2$, 1.5 mM $MnCl_2$, 0.1 mM EDTA, 50 mM NaCl, 20 mM Hepes, pH 8.0 and 25 µM of each NTPs (ATP, UTP, GTP and CTP) was incubated along with 2 µl of the test substance in 18 µl reaction mixture for 5 minutes in ice so as to bind the potential target at the active site of β-subunit of RNA polymerase. After 5 minutes 2 µl of pUC18 plasmid (250 µg/ml) was added. The reaction was continued for 30 min at 37°C.

2.9.2 PZase assay

2.9.2.1 PZase assay of clinical strains of *M. tuberculosis*

PZase activity of clinical isolates of *M. tuberculosis* was determined by Dr. Maria Helena SAAD, Fiocruz, Brazil) using a modified method described by Wayne (Wayne 1974). In brief, a heavy loopful of mycobacterial culture freshly grown on Lowenstein-Jensen medium was inoculated onto 5 ml of Dubos broth medium supplemented with 100 µg/ml PZA and 2 mg/ml sodium pyruvate in a 16 x 125 mm glass tube with screw cap. After incubation at 37°C for 4 or 7 days (when negative at the fourth day the tube was incubated for three more days), 1 mL of freshly prepared 1% ferrous ammonium sulphate solution was added to each tube, and the presence of a pink band was assessed. *M. tuberculosis* strain H37Rv, which is susceptible to PZA and positive for PZase, was used as a positive control and *M. fortuitum* was used as negative control for the assay. Strains with phenotypic and genotypic discordant results were retested for PZA susceptibility and PZase activity.

2.9.2.2 Cell free PZase assay

Enzymatic assay of PncA was done in 100 mM Glycine buffer pH 6.0 with 1-5 µM *M. tuberculosis* PncA, 25-500 µM PZA, and 1000 µM ammonium ferrous sulphate. The enzyme activity was monitored at 450 nm with UV mc2 Spectrophotomer using SP2000 v6.10.6.2 (SAFAS, Monaco). The positive reaction was characterized by the increment of OD_{450} due to production of orange-red complex formed by reaction of POA and ammonium ferrous sulphate (Allen et al. 1953).

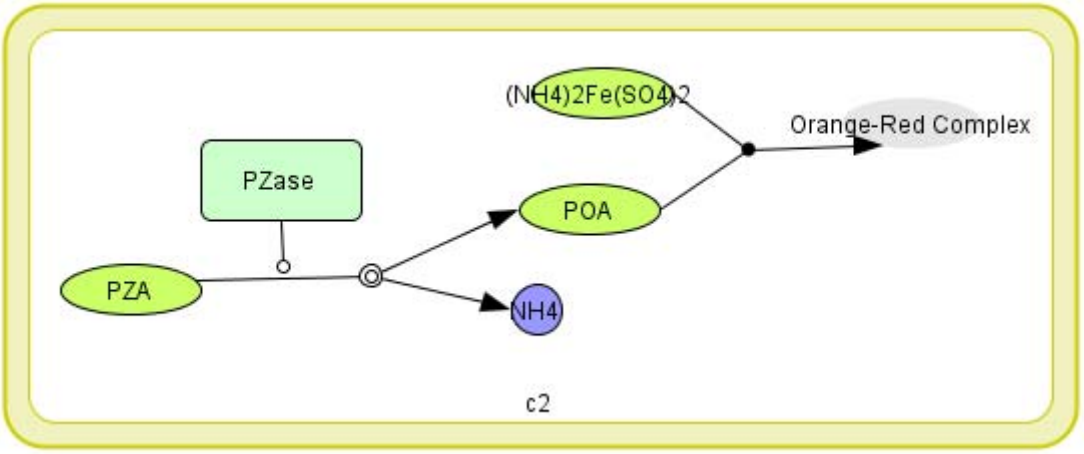


Fig. 9. Schematic Diagram showing the principle of detection of PZase assay.

2.9.2.3 Whole cell PZase assay with *pncA* mutants developed in this study

PZase assay with recombinant *pncA* clones in *E.coli* (wild type and mutant) was done by a modification as described by Wayne (Wayne 1974). The cells were grown overnight in LB media supplemented with 50 µg/ml kanamycin overnight. They were inoculated in 2.5 ml LB media pH 5.5 supplemented with 50 µg/ml kanamycin & 100 µg/ml PZA. They were grown from OD_{600} 0.2 for 3 hrs at 37°C without shaking. 0.5 ml of 1% ammonium ferrous sulphate solution was added in each tube to view the result. The positive PZase reaction was observed by the development of orange-red colour in the media immediately after the addition of ammonium ferrous sulphate.

Alternatively in a micro scale experiment, 200 µl LB media supplemented with kanamycin and PZA was used in 96 well plate. 4 µl of overnight culture was used as inoculum and the plates were incubated at 37°C for 3 hours. PZase reaction was visualized by adding 40 µl of 1% ammonium ferrous sulphate solution.

2.9.2.4 High throughput cell free PZase assay developed during this study

The *pncA* mutants in *E. coli* BL21 (DE3) were cultured in APS-Kan plates at 18°C for 3 days. The cell mass was collected with a sterile toothpick in a 96 well plate. The cells were lysed with BugBuster (Novagen) or ultrasonication. Protein concentration of the samples were determined and the value was feed in Biomeck 2000 robot. The robot was programmed to pipette 10 µg of the protein from the samples in a 96 well plate containing 50 µl 100mM Glycine pH 6.0. PncA reaction was initiated by adding 50 µl of 100mM Glycine.HCl pH 6, containing 1 mM PZA and 2 mM ammonium ferrous sulfate. OD was measured at 450 nm with MRXTC Revelation (DYNEX) at the interval of 20 sec for 20 minutes.

2.9.2.5 Coupled enzymatic amidase assay

Coupled amidase (Nicotinamidase or Pyrazinamidase) assay was done as described by Boshoff (Boshoff and Mizrahi 1998). Briefly, the assay mixture consisted of 30 mM Tris-HCl pH 7.5 with 11 U glutamate dehydrogenase, 800 µM α-keto-glutaratic acid, 160 µM NADPH, 300 µM PZA or 300 µM nicotinamide and 5 µM of protein in 750 µl. The reaction was started with substrate either nicotinamide or PZA. The enzyme activity was monitored at 340 nm with UV mc2 Spectrophotomer using SP2000 v6.10.6.2 (SAFAS, Monaco). The positive reaction was characterized by the decrement of OD_{340} due to oxidation of NADPH to NADP.

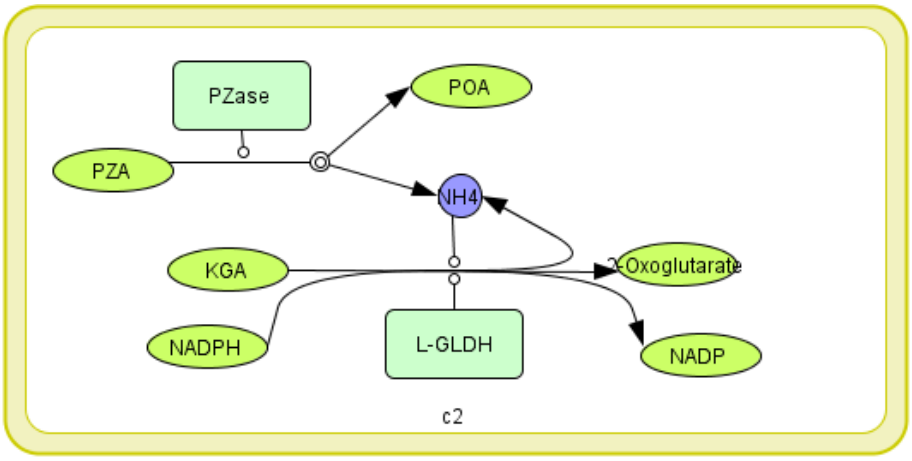


Fig. 10. Systematic Representation of coupled enzyme assay of PZase.

2.10 Bioinformatics

2.10.1 DNA sequence analysis

For the analysis of DNA sequences, the reading of raw sequence was done with FinchTV (Geospitza). BLAST at NCBI (http://blast.ncbi.nlm.nih.gov) was used for searching nucleotide sequences.

For the analysis of mutations, the DNA sequences obtained from the DNA sequencer 3730xl DNA Analyzer (Applied Biosystems) were analysed using the Pregap v1.6 and Gap4 v4.11 of the Staden Package (Staden et al. 2000).

2.10.2 Molecular modelling

Molecular Modelling was done with CellDesigner ver 3.5.1

2.11 Crystallization trials

Crystallization trials were done with the purified proteins in 96 well screen plates. The purified protein was concentrated up to 5-10 mg/ml. 2 µl of the purified protein was pipetted in the well of crystallization plate. The reservoir contains buffers. Buffers used were from Crystal Screen and Crystal Screen 2 (Hampton) was pipetted in the reservoir.

Also Mosquito Nanodrop Crystallization robot (TTP LabTech) was used for preparing the 200 nl protein drops on pre-filled crystallisation plates. In case of PncA, either protein alone or together with PZA (substrate) or POA (product), were used in the well contains 65 µl of Buffers of PACT Suite (Qiagen) or JSGC+ Suite (Qiagen). The plates containing proteins and buffers were left in incubator at 20°C undisturbed. The plates were observed after a day, after a week and after a month under light microscope for the development of crystal.

3 Results

Objective: Evaluation of current assays for RNAP and PncA.

3. 1. RNA Polymerase as drug target

3.1.1 Evaluation of current fluorescent assay

As one of a drug target the β-subunit of RNAP is well known. The fluorescent assay described by Kozlov (Kozlov et al. 2005) was attempted for establishing at HZI for screening of corallopyronin like substances. However with time it was realized that the assay was not so simple as it was reported. The main problems encountered were inability of RNAP to utilize substrate (modified nucleotide) and the self fluorescence of the substrate, which resulted in high background. Thus making it impossible to implement the assay, though the substrates were purified with HPLC. Therefore alternative assay for RNAP activity had to be established (see below).

3.1.2 Development of novel RNAP assay using *E. coli* RNAP

A pre-requisite for HTS is a simple and robust assay for the drug target. Our logic for developing the new assays was based on the observation that PPi is released during *in vitro* transcription of RNA. What we needed was an efficient and HTS compatible additional test that could quantitate the released PPi without the use of a radioactive substance. Based on our knowledge of Pyrosequencing, we reasoned that Sulfurylase could convert PPi to ATP, which in turn can be detected with the help of standard luciferin / luciferase assay. Our strategy is shown diagramatically below in figure 9.

Principle of the new Assay:

$$\text{DNA + NTP} \xrightarrow{\text{RNA Polymerase}} \text{DNA + RNA + PPi}$$

$$\text{ATP} \xrightarrow{\text{Apyrase}} \text{ADP + Pi}$$

$$\text{PPi + APS} \xrightarrow{\text{ATP sulfurylase}} \text{ATP}$$

$$\text{ATP + Luc} \xrightarrow{\text{Luciferase}} \text{Oxyluciferin + hv}$$

NTP= Nucleotides (ATP, UTP, GTP& CTP)
PPi= Pyrophosphate
APS= adenosine 5´ phosphosulfate
Luc= Luciferin

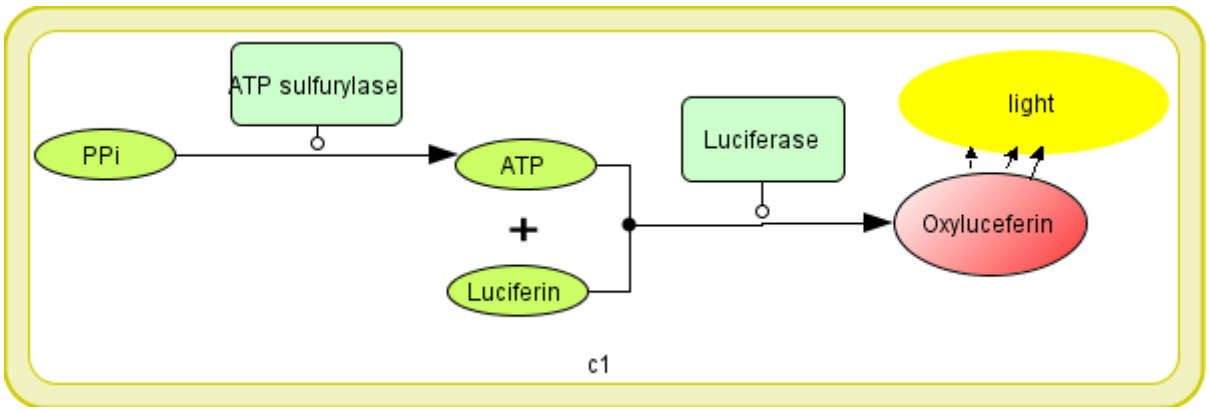


Fig. 11. Schematic diagram showing the principle of the new RNAP Assay developed in this study.

3.1.2.1 Validity of the assay

The assay was tested for the validity of method. First it was checked if the measurement of ATP alone could be done properly with the Luciferin-Luciferase system as the first standard method.

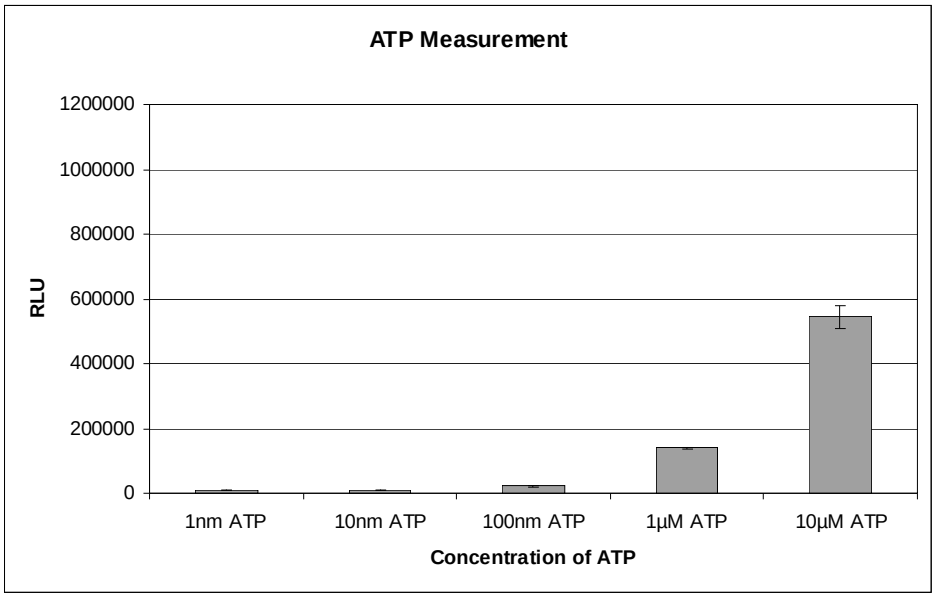


Fig. 12. Test of Luciferin-Luciferase system. RLU measured with defined concentration of ATP (1 nM to 10 µM) with Luciferin-Luciferase System. The reaction mix contains various concentration of ATP from 1 nM to 10 µM and Luciferin-Luciferase system.

After the measurement of the ATP concentration gradient, the ability of ATP Sulfurylase to convert PPi to ATP was determined by using PPi as the substrate. In positive reaction 10µM of PPi was incubated with 5 µM adenosine-5´-phosphosulfate and 30 mU ATP Sulfurylase. PPi was excluded in the negative reaction. The reaction was carried out for 10 min at 30°C during which ATP sulfurylase converts PPi to ATP in expense of adenosine 5´ phosphosulfate. A final denaturating of the enzymes present in the whole reaction was performed at 85°C for 10 min. Finally net ATP produced during the whole reaction was evaluated with ATP kit.

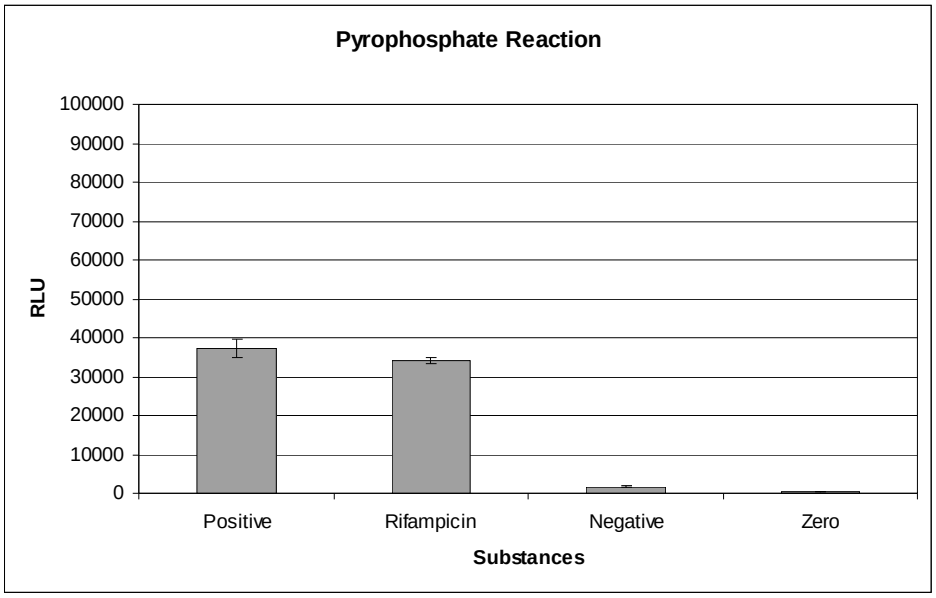


Fig. 13. Test of pyrophosphate reaction with the Luciferin-Luciferase system. In the positive reaction PPi, was reacted with ATP sulfurylase, which was then subjected to Luciferin-Luciferase system and RLU values measured. Rifampicin contains rifampicin in the positive reaction. In negative reaction PPi was excluded and Zero was the blank value measured.

As the test done with PPi reaction gives positive result, final RNAP assay was done with reaction starting from RNAP. The RNAP assay as a whole is a successive enzyme assay containing four different enzymatic reactions.

The complete RNAP assay was already described in previous sections.

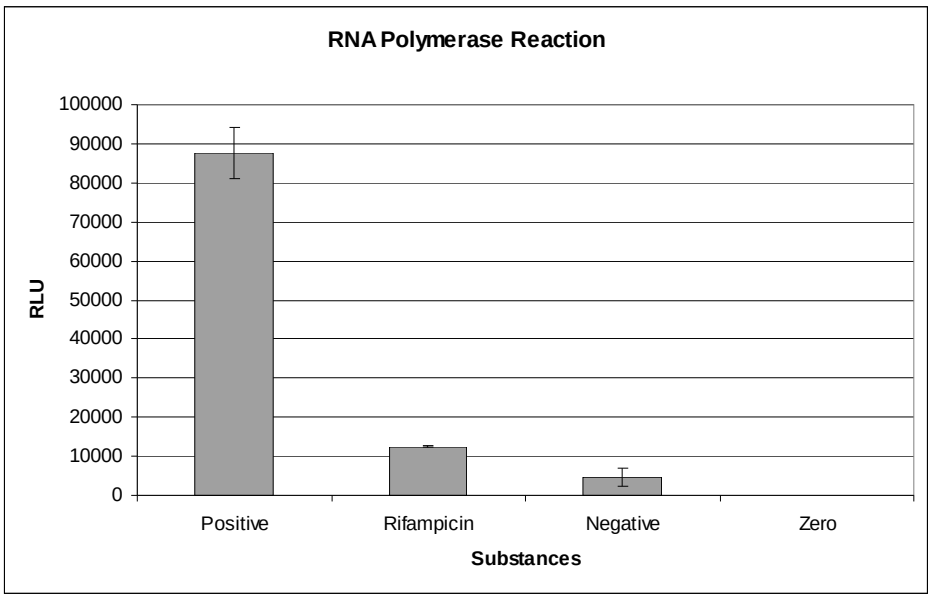


Fig. 14. Positive reaction contain all reactants necessary for RNAP Assay. Rifampicin contained Rifampicin, a known inhibitor of RNAP. Negative reaction did not have DNA necessary for the transcription of RNAP and Zero was the blank well.

The Fig. 15 shows the measurement of RLU upon in vitro transcription using pUC18 DNA template and subsequent reactions. The controls without DNA or RNAP show the luminescence value of the controls lacking DNA or RNAP. Other negative controls done were exclusion of both DNA and RNAP or using heat denatured RNAP. With these negative controls also the RLU measured in RNAP assay was comparable to the negative controls where DNA or RNAP were excluded from the assay.

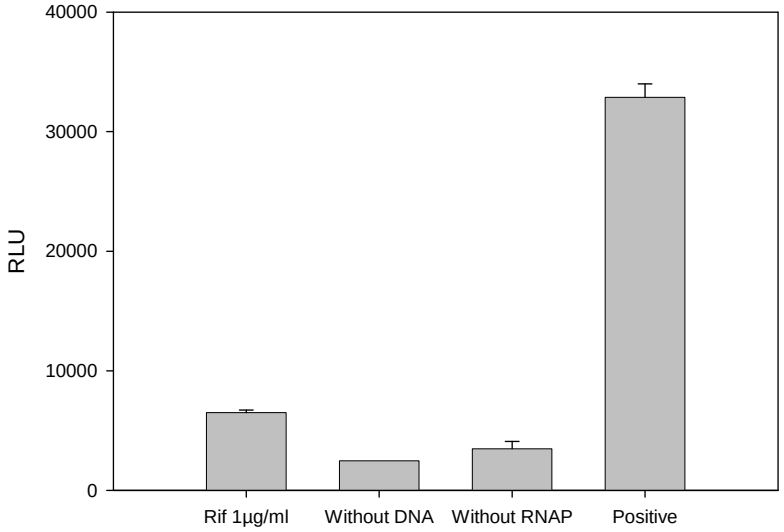


Fig. 15. RNAP Assay with Negative controls (without DNA or without RNAP) and known inhibitor Rifampicin. Luminescence signal detected at different conditions. Positive, complete reaction mixture; Rif 1 µg/ml complete reaction mixture with rifampicin 1µg/ml; Without DNA, complete assay excluding pUC18; Without RNAP, complete assay excluding RNAP.

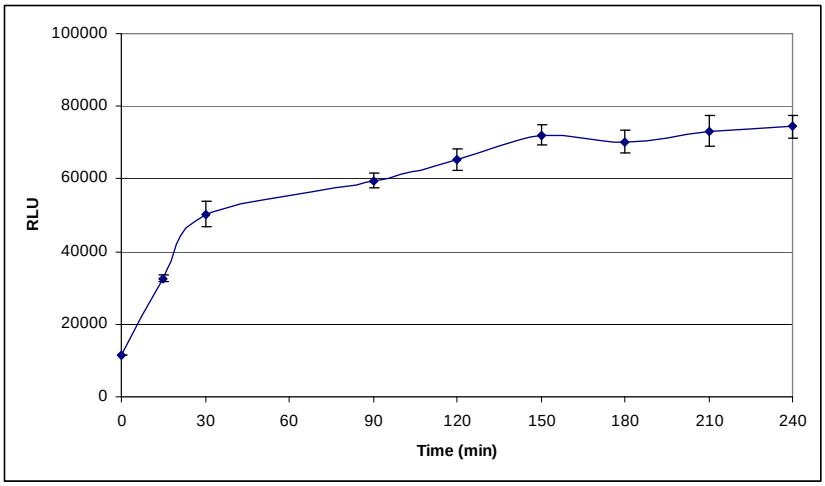


Fig. 16. Time dependent increment of RLU value.

The RNAP assay was tested by carrying out after various durations of transcription. A steady increase of RLU value was observed.

The RNAP assay was tested with rifampicin, corallopyronin, sorangicin and ripostatin, which are all known inhibitor of RNAP. The result showed that all of them inhibit the RNAP. Fig. 17 shows the inhibiting effect on RNAP activity exerted by rifampicin and corallopyronin, typical inhibitors of RNAP, which was detected with the RNAP assay. The extent of inhibition was related to the concentration of inhibitor used. The majority of RNA synthesis on pUC18 is expected to originate at the *bla* promoter where the 5′ terminus with sequence pppGAUAAAUG (Kozlov et al. 2005).

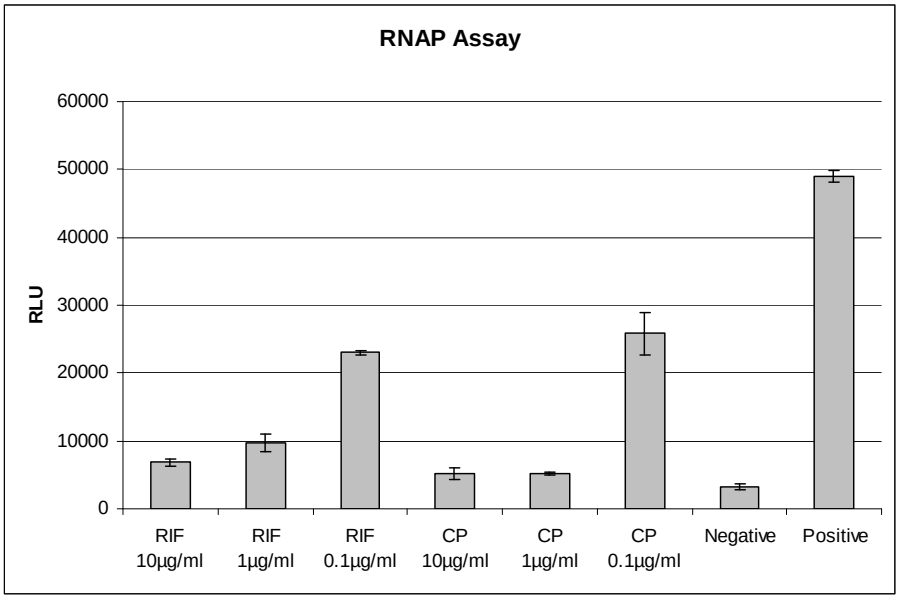


Fig. 17. Concentration dependent inhibition observed in RNAP assay. Rifampicin (RIF) at three concentrations and corallopyronin (CP) at three different concentrations are shown.

3.1.2.2 Robustness of the assay

The quality performance and the robustness of an assay is defined by the Z´-factor (Zhang et al. 1999), which is calculated from the assay signal-to-noise ratio and the signal to background ratio. The statistical measure for the robustness of the assays is defined by the following equation:

$$Z' = 1 - \frac{(3\sigma_{(+)} + 3\sigma_{(-)}}{|\mu_{(+)} - \mu_{(-)}|}$$

where $\mu_{(+)}$ and $\sigma_{(+)}$ are the mean SD for the positive reaction, respectively, and $\mu_{(-)}$ and $\sigma_{(-)}$ are the mean and SD for the background signal, respectively, and the denominator term is the absolute value of the difference in the means of the two measurements. The maximum value of Z´ is 1 for an ideal assay, where the signal and background do not deviate from their mean value. For a practicable assay, value ≥ 0.5 are acceptable for high throughput library screening. The Z´ statistic is a general measure of assay robustness and is also applied to any other enzymatic or other assay.

The Z´values calculated in the RNAP assay was in the range of 0.758 to 0.901.

Objective : Screening potential drug candidates using the optimized assay for RNAP.

3.1.2.3 Screening potential drug candidates using the new assay for RNAP

Result obtained from the assay including Z´-factor showed it to be suitable for determining RNAP activity for HTS. To optimize the assay for the HTS, robot Biomek 2000 was used with software Bioworks package v 3.5 (Beckmann Instruments, Inc) to pipette the assay components in 96 well plate. The assay in 386 plate was technically limited due to the cross talk of adjacent walls when measuring luminescence value.

Complete inhibition was taken for the RNAP assay where there was no inhibitors or other substance. Null inhibition was taken where RNAP did not polymerise in the reaction i.e. either without DNA or without RNAP or with heat inactivated RNAP.

In each of the experiments three samples were used to determine the SD of the measurement.

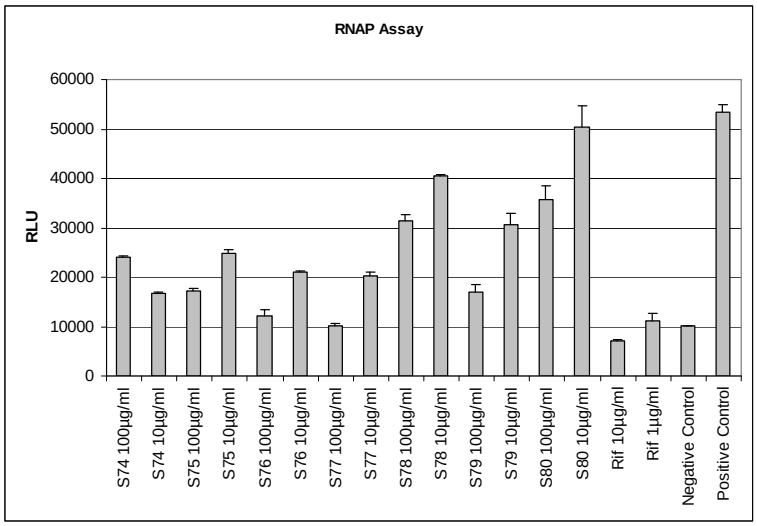


Fig. 18. Screening of drug candidates by the new RNAP assay. RNAP assay was performed with different substances at 100 µg/ml and 10 µg/ml along with positive control which was reaction with all substance, negative control where DNA was excluded and two rifampicin concentrations of 1 µg/ml and 10 µg/ml as control of RNAP inhibition. The Y-axis shows the RLU measured after the reaction and at the X-axis the substances are shown.

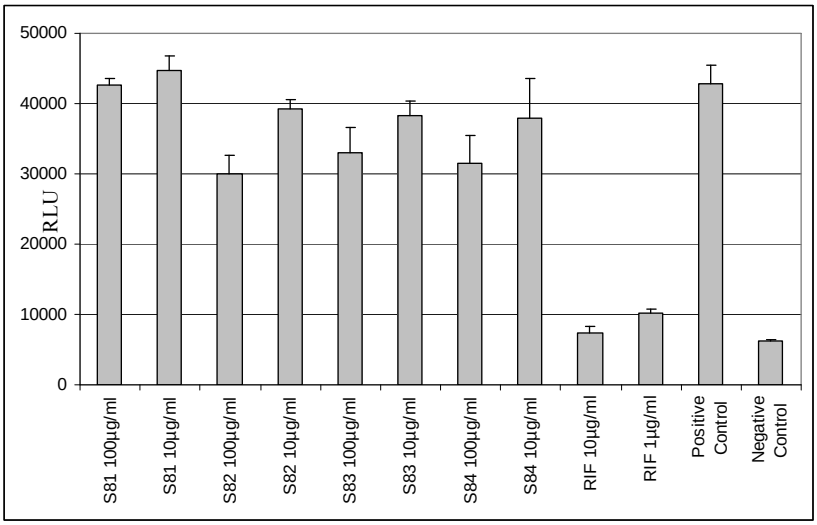


Fig. 19. As described in the legend for Table 35, different substances were tested at a concentration of 100 µg/ml and 10 µg/ml. Positive control was the reaction with all substance. DNA was excluded in Negative control. Rifampicin at a concentration of 1 µg/ml and 10 µg/ml were used as controls for RNAP inhibition. The Y-axis shows the RLU measured after the reaction and at the X-axis the substances are shown.

Table 34. Comparisons of inhibition achieved observed by Kirby-Bauers method and RNAP assay method for the inhibition of known RNAP inhibitors.

RNAP Inhibitors	Zone of Inhibition in *E. coli* DH5α with 10µg Subst. (Disc Diffusion Method)	RNAP Inhibition at conc. 1 µg/ml	RNAP Inhibition at conc. 0.1 µg/ml
Rifampicin	0-1mm	96% ± 10%	78% ± 10%
Corallopyronin	NA	96% ± 20%	47% ± 15%
Sorangicin	0-2mm	78%	78%
Ripostatin	0-2mm	80%	44%

Table 35. Comparisons of inhibition observed by Kirby-Bauers method and the new RNAP Assay for the inhibition of the test substances.

Substances	Name	Zone of Inhibition in *E. coli* DH5α with 10µg Subst. (Disc Diffusion Method)	RNAP Inhibition at conc. 10µg/ml	RNAP Inhibition at conc. 100µg/ml
Substance 16	CP 14a	2-3mm	20% ± 1%	24% ± 3%
Substance 17	CP-17	≤ 3mm	21% ± 1%	26% ± 8%
Substance 18	CP-18	≤ 4mm	17% ± 3%	19% ± 3%
Substance 19	CP-19	≤ 4mm	16% ± 2%	18% ± 4%
Substance 21	CP-21a	≤ 3mm	20% ± 5%	33% ± 5%
Substance 24	CP-24a	≤ 4mm	20% ± 3%	25% ± 5%
Substance 25	CP-25a	2-3mm	19% ± 2%	15% ± 2%
Substance 28	CP-28a	2-3mm	19% ± 4%	20% ± 4%
Substance 30	CP-30	1-2mm	18% ± 4%	20% ± 3%
Substance 55	GW 387	1-2mm	18% ± 3%	20% ± 4%
Substance 56	GW 386	1-2mm	17% ± 3%	22% ± 6%
Substance 57	GW 389	1-2mm	23% ± 4%	37% ± 9%
Substance 58	GW 341	≤ 1mm	21% ± 5%	47% ± 12%
Substance 59	GW 342	1-3mm	24% ± 5%	35% ± 7%
Substance 60	CP40	≤ 1mm	20% ± 4%	25% ± 1%
Substance 61	CP 43	≤ 1mm	20% ± 3%	42% ± 13%
Substance 62	CP 44	≤ 1mm	19% ± 3%	29% ± 3%
Substance 63	CP45	≤ 1mm	21% ± 2%	34% ± 7%
Substance 64	CP46	≤ 1mm	19% ± 3%	26% ± 7%
Substance 65	CP47	≤ 1mm	19% ± 2%	30% ± 8%
Substance 66	CP48	0mm	22% ± 3%	31% ± 7%
Substance 71	GW414	1-2mm	24% ± 3%	35% ± 7%
Substance 74	CP 57	≤ 1mm	85% ± 1%	68% ± 0%
Substance 75	CP 58	≤ 1mm	66% ± 2%	83% ± 1%
Substance 76	CP 59	0-2mm	75% ± 0%	95% ± 3%
Substance 77	CP 60	≤ 1mm	76% ± 2%	100% ± 1%
Substance 78	CP 61	?mm	30% ± 1%	51% ± 3%
Substance 79	CP 63	NA	53% ± 6%	84% ± 4%
Substance 80	CP 64	1-2mm	7% ± 10%	41% ± 6%
Substance 81	CP-66	NA	1% ± 2%	-5% ± 5%
Substance 82	CP 68	NA	35% ± 6%	10% ± 3%
Substance 83	CP 69	NA	27% ± 8%	12% ± 5%
Substance 84	CP70	NA	31% ± 9%	13% ± 13%

From all the tested substance, the substances 74, 76 and 77 seemed to be promising candidates as these show reasonable inhibition of RNAP (85%, 75% and 76%, respectively at 10 µg/ml). These corallopyronin like substances might be potential

substances for the future optimization for the development of new drugs for rifampicin resistant *M. tuberculosis*.

3.1.3 Attempt to reconstitute *M. tuberculosis* RNAP holoenzyme *in vitro*

Objective: Attempt to reconstitute *M. tuberculosis* RNAP holoenzyme for use in drug screening.

The results described above were all done using *E.coli* RNAP. One of the objectives of this work was to produce RNAP components of *M. tuberculosis* in *E.coli* and then attempt to reconstitute the holoenzyme which could then be used for drug screening. In our view such an assay would be better suited for drug development for *M. tuberculosis* in future.

3.1.3.1 Cloning, expression and purification of RpoA, RpoB, RpoC and RpoZ

The complete genes (*rpoA*, *rpoB*, *rpoC* and *rpoZ*) were amplified from *M. tuberculosis* H37Rv DNA using the PCR primer sets for the gene. The PCR products were cleaved and extracted which serve as DNA template for the next cloning primer. Cloning primers containing restriction sites were used for the re-amplification, which were used for the ligation in pET vectors (Novagen). Transformants were selected in LB agar with appropriate antibiotics. The transformants with insert were determined by checking the over-expression of the protein in APS agar (auto induction). Alternatively transformants with insert were also determined by colony PCR. Nucleotide sequences of the insert in pET vector were determined by conventional DNA sequencing and the identification of the protein was confirmed by protein sequencing. All the genes were cloned with and without histidine tag. Histidine tagged RpoA, RpoB and RpoZ were purified using a series of chromatography from Ni-NTA affinity chromatography to Q-Sepharose ion-exchange chromatography and finally with Sephadex-200 size exclusion chromatography.

Table 36. PCR condition for the amplification of *rpoA* (1044 bp). Qiagen Master Mix was used for the PCR.

95°C	10 min	
95°C	30 sec	
55 °C	30 sec	30x
72°C	1 min	
72°C	10 min	
4°C	forever	

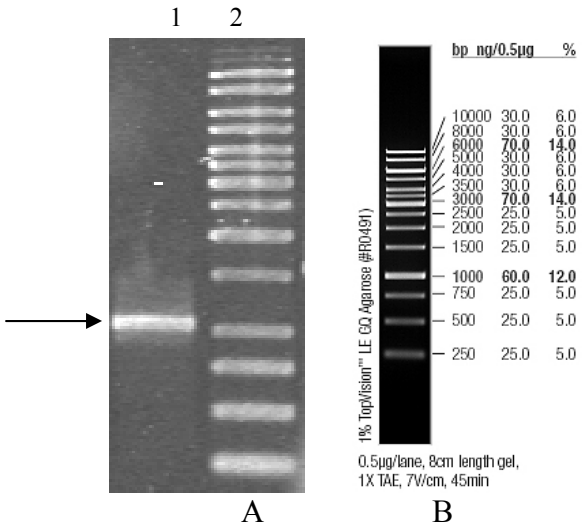


Fig. 20 A. 1.5% Agarose gel showing PCR amplified product of *rpoA* (arrow shows expected size of 1176 bp) from *M. tuberculosis* H37Rv. Lanes: 1, PCR product; 2, GeneRuler™ 1kb DNA Ladder.

Fig. 20 B. GeneRuler™ 1kb DNA Ladder (Fermentas).

The amplification of *rpoA* from *M. tuberculosis* H37Rv DNA was done which was at the expected size of 1176 bp as shown with an arrow in Fig. 20 A.

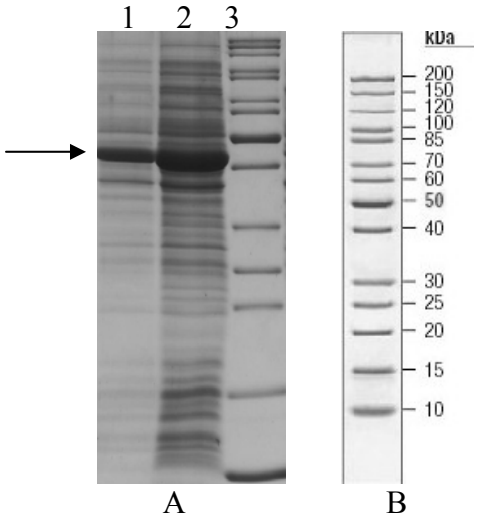


Fig. 21 A. SDS-PAGE of *M. tuberculosis* RpoA-His (C-terminal His-tagged) expressed in *E. coli* BL21 (DE3). Lanes : 1, Insoluble extract after 3 hours of induction with IPTG; 2, Soluble extract after 3 hours of induction with IPTG; 3, PageRuler™ Protein Ladder. Rpo expected size: 38.5 kDa (arrow).

Fig. 21 B. PageRuler™ Protein Ladder (Fermentas).

The expression of recombinant C-terminal histidine tagged RpoA was done which was at the expected band of 38.5 kDa as indicated with an arrow in Fig. 21 A.

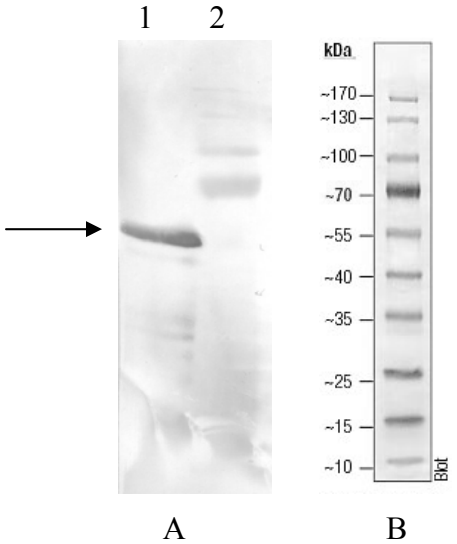


Fig. 22 A. Western Blotting of the *M. tuberculosis* RpoA-His. Lanes: 1, His-tagged *M. tuberculosis* RpoA-His (positive reaction shown with an arrow); 2, PageRuler™ Prestained Protein Ladder (Fermentas).

Fig. 22 B. PageRuler™ Prestained Protein Ladder (Fermentas).

Fig. 22 A shows the result of western blotting to detect histidine tag. The result confirmed that the protein was histidine tagged as indicated with an arrow.

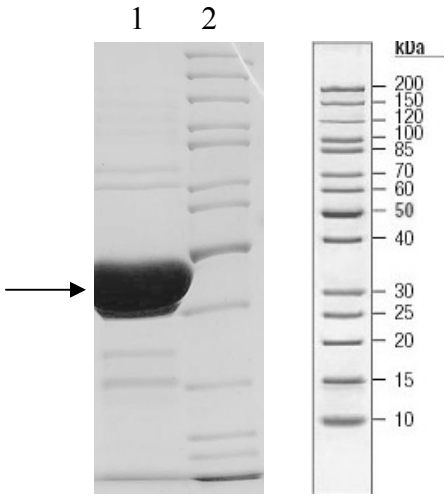


Fig. 23. SDS-PAGE of purified *M. tuberculosis* RpoA-His from *E. coli* BL2 (DE3). Recombinant his-tagged RpoA was purified with a series of chromatography starting from affinity chromatography (Ni-NTA), ion-exchange chromatography (Q-Sepharose) and finally with gel exclusion chromatography (Superdex-200) to yield a homogenous protein of 38.5 kDa as indicated with an arrow in Fig. 23 A.

Lanes: 1, Purified *M. tuberculosis* RpoA-His component; 2, PageRuler™ Protein Ladder (Fermentas).

Fig. 23 B. PageRuler™ Protein Ladder (Fermentas).

Recombinant his-tagged RpoA was purified with a series of chromatography starting from affinity chromatography (Ni-NTA), ion-exchange chromatography (Q-Sepharose) and finally with gel exclusion chromatography (Superdex-200) to yield a homogenous protein of 38.5 kDa as indicated with an arrow in Fig. 23A.

Table 37. PCR condition for the amplification of *rpoB* (3519 bp). Takara LA was used for the amplification of *rpoB*.

95°C	10 min	
95°C	30 sec	
49 °C	30 sec	35x
72°C	3.5 min	
72°C	10 min	
4°C	forever	

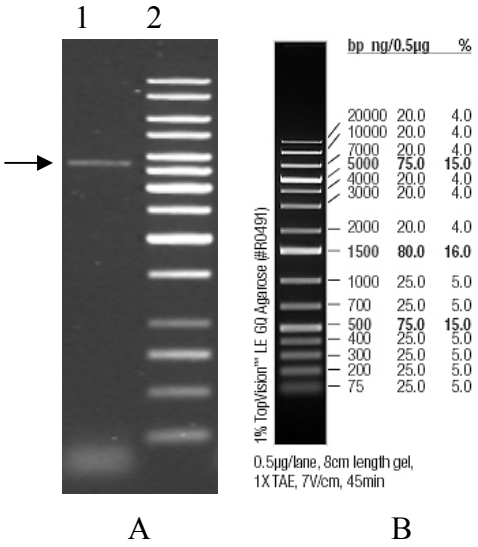


Fig. 24 A. 1% Agarose gel showing PCR amplified product of *rpoB* from *M. tuberculosis* H37Rv (expected size of 3527 bp shown with arrow) . Lanes: 1, PCR product; 2, 100bp GeneRuler™ 1kb DNA Ladder Plus (Fermentas).

Fig. 24 B. GeneRuler™ 1kb DNA Ladder (Fermentas).

Similarly, amplification of *rpoB* from *M. tuberculosis* H37Rv DNA was done with Takara LA which was at the expected size of 3527 bp as shown with an arrow.

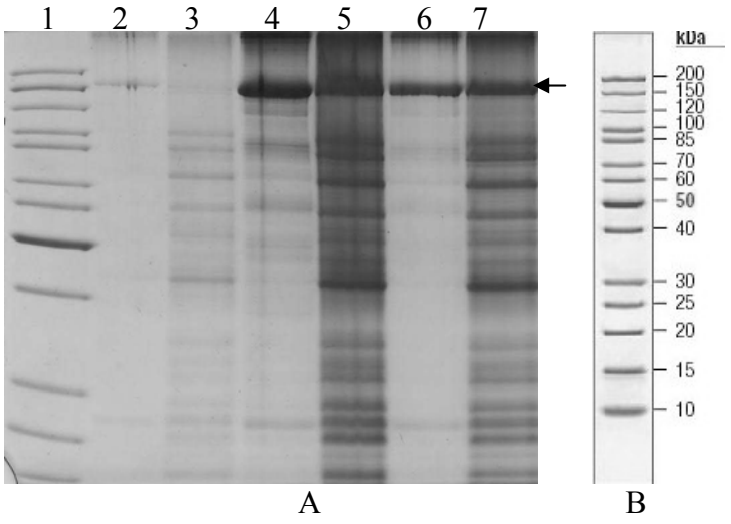


Fig. 25 A. SDS-Page of *M. tuberculosis* His-RpoB (N-terminal His-tagged) expressed in *E. coli* BL21 (DE3). Lanes: 1, PageRuler™ Protein Ladder; 2, Pre-induced soluble extract; 3, Pre-induced insoluble extract; 4, Soluble extract after 3 hours of induction with IPTG; 5, Insoluble extract after 3 hours of induction with IPTG; 6, Soluble extract after overnight of induction with IPTG; 7, Insoluble extract after overnight of induction with IPTG.

Fig. 25 B. PageRuler™ Protein Ladder (Fermentas).

Expression of recombinant RpoB was done in LB medium (Fig. 25 A) and in APS agar (Data not shown). In the Fig. 25 A, gene expression induced with IPTG was shown at two different time intervals; 3 hours after induction and overnight after induction.

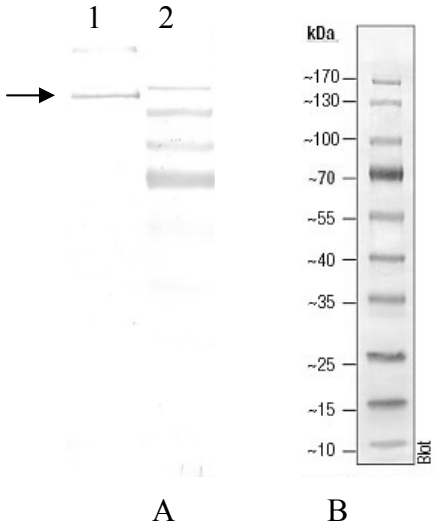
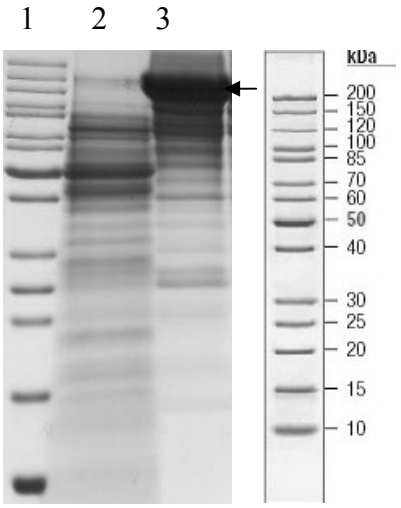


Fig. 26 A. Western Blotting of the *M. tuberculosis* His-RpoB tagged with His. Lanes: 1, Positive His-tagged; 2, PageRuler™ Prestained Protein Ladder.

Fig. 26 B. PageRuler™ Prestained Protein Ladder (Fermentas).

The result of western blotting to detect histidine tag showed that the protein was histidine tagged as indicated with an arrow in Fig. 26 A.



A B

Fig. 27 A. Purified *M. tuberculosis* His-RpoB (N-terminal His-tagged) expressed in *E. coli* BL21 (DE3). Lanes: 1, PageRuler™ Protein Ladder; 2, Degradation product of *M. tuberculosis* His-RpoB; 3, Purified *M. tuberculosis* His-RpoB.

Fig. 27 B. PageRuler™ Protein Ladder (Fermentas)

Recombinant his-tagged RpoB was purified with a series of chromatography starting from affinity chromatography (Ni-NTA), ion-exchange chromatography (Q-Sepharose) and finally with gel exclusion chromatography (Superdex-200) to yield a protein of 130 kDa as indicated with an arrow in Fig. 27 A. During the purification of this protein, degradation was main problem. The problem was solved by using buffer containing 10% Glycerol.

Table 38. PCR condition for the amplification of *rpoC* (3951 bp). Takara LA was used for the amplification of *rpoC*.

95°C	10 min	
95°C	30 sec	
52 °C	30 sec	30x
72°C	4 min	
72°C	10 min	
4°C	forever	

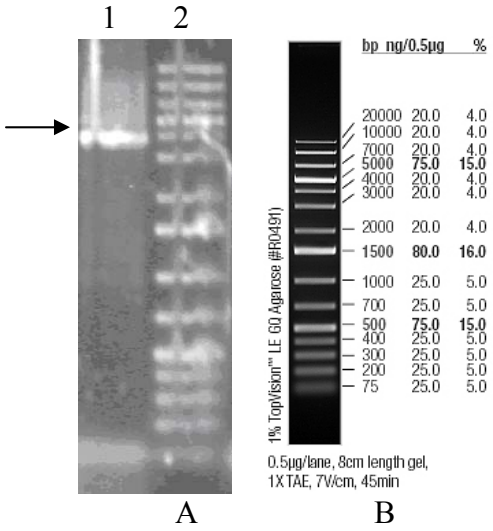


Fig. 28 A. 1% Agarose gel showing PCR amplified product of *rpoC* from *M. tuberculosis* H37Rv ,expected size of 4280 bp (arrow). Lanes: 1, PCR product; 2, GeneRuler™ 1kb DNA Ladder Plus (Fermentas).

Fig. 28 B. GeneRuler™ 1kb DNA Ladder Plus (Fermentas).

Similarly, amplification of *rpoC* from *M. tuberculosis* H37Rv DNA was done with Takara LA, which was at the expected size of 4280 bp as shown with an arrow. This gene could not be amplified only with this particular kit.

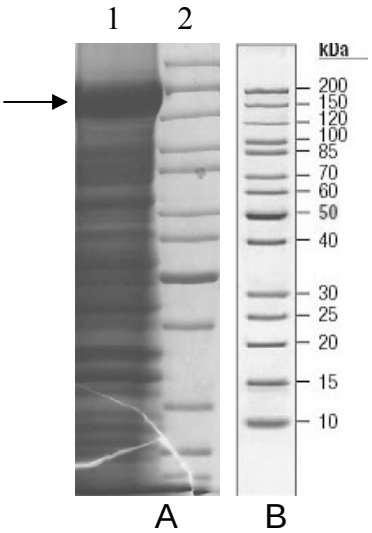


Fig. 29 A. SDS-Page. *M. tuberculosis* RpoC expressed in *E. coli* BL21 (DE3) in APS medium. Lanes: 1, Auto induced soluble extract; 2, PageRuler™ Protein Ladder.

Fig. 29 B. PageRuler™ Protein Ladder (Fermentas)

The recombinant RpoC was expressed in APS kanamycin agar. The Fig. 29 A shows the induction of soluble fraction of the protein of expected size of 146.7 kDa as indicated with an arrow.

Table 39. PCR condition for the amplification of rpoZ (333 bp). Qiagen Master Mix was used for the amplification of rpoZ.

95°C	10 min	
95°C	30 sec	
52 °C	30 sec	30x
72°C	30 sec	
72°C	10 min	
4°C	forever	

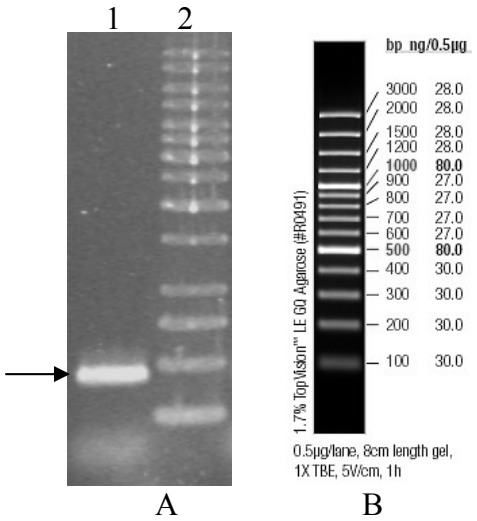


Fig. 30 A. 1% Agarose gel showing PCR amplified product of rpoZ from *M. tuberculosis* H37Rv. Lanes: 1, PCR product; 2, 100bp GeneRuler™ 100 bp DNA Ladder (Fermentas).

Fig. 30 B. 100bp GeneRuler™ 100 bp DNA Ladder (Fermentas).

Amplification of *rpoZ* from *M. tuberculosis* H37Rv DNA was done which was at the expected size of 439 bp as shown with an arrow.

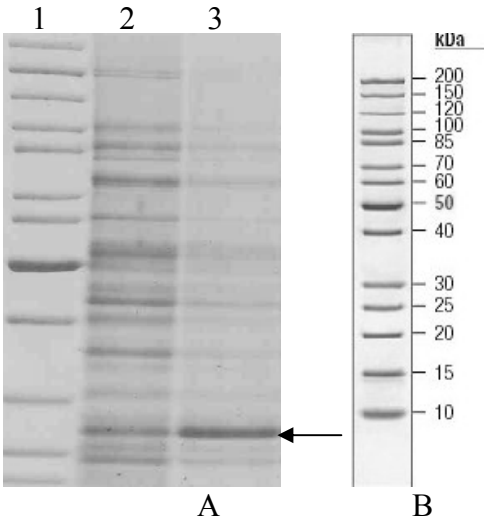


Fig. 31 A. SDS-PAGE of *M. tuberculosis* RpoZ-His (C-terminal His-tagged) expressed in *E. coli* BL21 (DE3) in APS medium at 18°C for 3 days. Lanes :1, PageRuler™ Protein Ladder; 2, Insoluble extract ; 3, Soluble extract.

Fig. 31 B. PageRuler™ Protein Ladder (Fermentas).

The recombinant RpoZ was expressed in APS kanamycin agar. The Fig. 31 A shows the induction of soluble fraction of the protein of expected size of 12.6 kDa as indicated with an arrow.

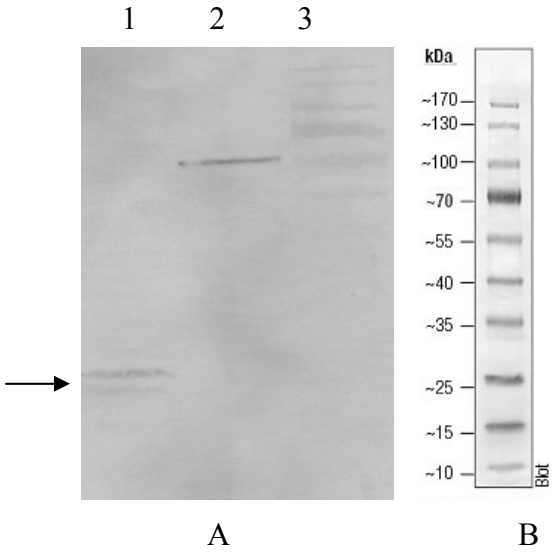
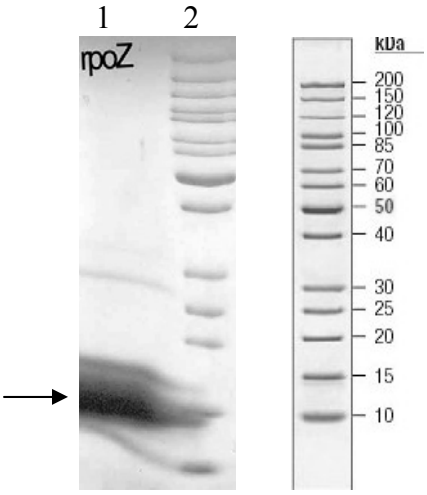


Fig. 32. A. Western Blotting of the *M. tuberculosis* RpoA-His. Lanes: 1, Positive His-tagged *M. tuberculosis* RpoZ-His; 2, Positive control; 3, PageRuler™ Prestained Protein Ladder

Fig. 34 B. PageRuler™ Prestained Protein Ladder (Fermentas)

The result of western blotting to detect histidine tag showed that the protein was histidine tagged as indicated with an arrow in Fig. 33 A.



A B

Fig. 34 A. SDS-PAGE of purified *M. tuberculosis* RpoZ-His from *E. coli* BL21 (DE3). Lanes:1, Purified *M. tuberculosis* RpoZ-His component; 2, PageRuler™ Protein Ladder (Fermentas).

Fig. 34 B. PageRuler™ Protein Ladder (Fermentas)

Recombinant C-terminal his-tagged RpoZ was purified with a series of chromatography starting from affinity chromatography (Ni-NTA), ion-exchange chromatography (Q-Sepharose) and finally with gel exclusion chromatography (Superdex-200) to yield a protein of 12.6 kDa as indicated with an arrow in Fig. 34 A.

3.1.3.2 Reconstitution of RNAP core enzyme

The attempt to reconstitute *M. tuberculosis* RNAP from its subunits was based on a previously described method using E. coli RNAP subunits (Tang et al. 1995).

3.1.3.2.1 Nondenaturing approach

C-terminal his-tagged RpoZ or C-terminal his-tagged RpoA was purified partially with Ni-NTA column. The other components of the RNAP without histidin-tag were suspended in Buffer A (20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole pH 7.8), the cells were lysed by sonication and the lysate was cleared by centrifugation (18000 rpm for 10 min at 4°C). Reconstitution of RNAP was done with one partially purified components viz C-terminal his tagged RpoA or C-terminal his-tagged RpoZ and mixed with rest of the soluble fraction components in the molar ratio of RpoZ, RpoA, RpoB and RpoC to 1:2:1:1.

The reconstitution mixture was dialysed twice against 750 ml of Buffer C (50 mM Tris-HCl, 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, 20% Glycerol) for 4 hours and overnight. The resulting mixture was then incubated at 30°C for 45 min, and then cleared by centrifugation (18000 rpm for 10 min at 4°C). The mixture was stored at –20°C.

Reconstitution mix after dialysis was repurified in Ni-NTA column, then concentrated in Vivaspin 20 with 100 kDa MWCO to remove unbound His-tagged components from the protein solution. Buffer exchange was also done at the same time.

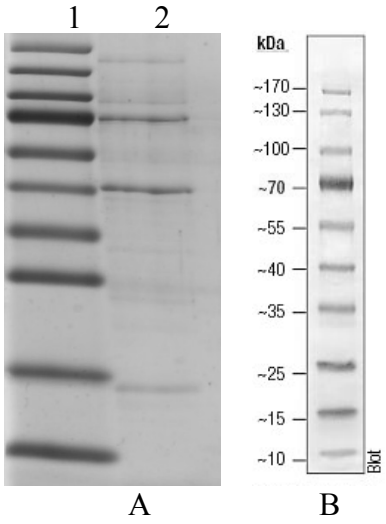


Fig. 35. Reconstituted RNAP from mixture of RNAP components of *M. tuberculosis*. Lane 1: PageRuler™ Prestained Protein Ladder

Fig. 35.B. PageRuler™ Prestained Protein Ladder (Fermentas).

The reconstituted RNAP did not show any activity in the RNAP assay as compared to the *E.coli* RNAP.

3.1.3.2.2 Denaturing approach

The partially purified RpoA or RpoZ tagged with histidine were used in conjunction with other subunits of RNAP. The cell mass which over express RpoA and RpoZ were suspended in Buffer A and the cells were lysed by sonication and the lysate was cleared by centrifugation. The cell pellets (RpoC, RpoB) were resuspended in Buffer D (8 M Urea, 50 mM Tris-HCl, 10 mM MgCl2, 10 µM ZnCl2, 1 mM EDTA, 10 mM DTT, 10% Glycerol pH 7.8) to lyse the cells. The lysates were centrifuged at 13000 rpm for 15 min at 4°C. For the purpose of reconstitution partially purified component either RpoA or RpoZ was mixed with the rest of the fraction components in the molar ratio of RpoZ, RpoA, RpoB and RpoC to 1:2:1:1.

The reconstitution mixture was dialysed against 750 ml of Buffer C (50 mM Tris-HCl, 200 mM KCl, 10 mM $MgCl_2$, 10µM $ZnCl_2$, 1mM EDTA, 5mM 2-mercaptoethanol, 20% Glycerol) for 2 times. The resulting mixture were next incubated at 30°C for 45, and then cleared cleared by centrifugation (18000 rpm for 10min at 4°C). The mixture was stored at –20°C.

Reconstitution mix after dialysis was repurified in Ni-NTA column, then concentrated in Vivaspin 20 with 100kD MWCO to remove unbound His-tagged components from the protein solution. Buffer exchange was also done at the same time.

With the reconstituted RNAP, RNAP assay described at 2.9.1.2 was done. On comparison to *E. coli* RNAP, the reconstituted RNAP did not show any activity.

3.1.4 Mutation analysis of *rpoB* in clinical strains of *M. tuberculosis*

We also charcterised the clinical *M. tuberculosis* strains from Brazil regarding mutations in rpoB gene as described below

The *rpoB* gene from the clinical strains of tuberculosis of 6 rifampicin resistant and *M. tuberculosis* H37Rv were amplified using primers rpoB-F1 and rpoB-F2. The PCR products of 3527 bp were extracted with PEG precipitation method. The samples were given for sequencing along with PCR primers and rpoB sequencing primers. The raw sequences were aligned and joined using Pregap v1.6 and Gap4.11 of the Staden Package (Staden et al. 2000). All rifampicin resistant contain mutation restricted to a 69-bp region. As it would be unnecessarily to sequence the complete gene, only the initial (ca. 600 bp) and final part of *rpoB* (ca. 600 bp) was sequenced only with rpoB-F1 and rpoB-F2. There was no mutation present at the beginning or ending of the gene except the nonsense mutation D184D in 10 strains.

Table 40. Nucleotide changes detected in rpoB gene in clinical strains of *M. tuberculosis*.

Strain No.	RIF Sensitivity *	Position and mutation	Amino acid level
24	R	310 C>T, 526 C> T	D184D, H526Y
37	R	1299 C>T, 1304A>T	514 F, D516V
35	R	1303-4 GA>TT	D516F
25	R	1304A>T	D516V
32	R	1333C>T	H526Y
33	R	1349C>T	S531L
57**	S	310 C>T	D184D
8**	S	D516V	D184D
27**	S	D516V	D184D
31**	S	D516V	D184D
65**	S	D516V	D184D
73**	R	D516V	D184D
78**	S	D516V	D184D
82**	R	D516V	D184D
87**	S	D516V	D184D
89**	S	D516V	D184D

* Data provided from Dr. M.H Saad.

** Complete *rpoB* gene not sequenced.

3.2 Pyrazinamide resistance and pyrazinamidase

Objective: Identify mutation in pncA gene which are responsible for pyrazinamide resistance.

3.2.1 Mutation analysis of *pncA* in clinical strains of *M. tuberculosis*

The *pncA* gene from the clinical strains of tuberculosis was amplified with pncA-F2 and pncA-R2 primers. The DNA fragment up to 454 bp upstream of *pncA* was amplified with primers Rv2044cF and Rv2044cR. The PCR products were extracted with PEG precipitation method (2.6.4.2) and subjected to DNA sequencing. The raw sequences were aligned then joined using Pregap v1.6 and Gap 4.11 of the Staden Package (Staden et al. 2000).

The *pncA* gene of 24 out of 93 clinical strains contained mutations at different sites distributed throughout the *pncA* gene (Fig. 36). Four types of mutations were observed. Point mutations, insertion, transposase insertion and deletion which are shown in figure 35.

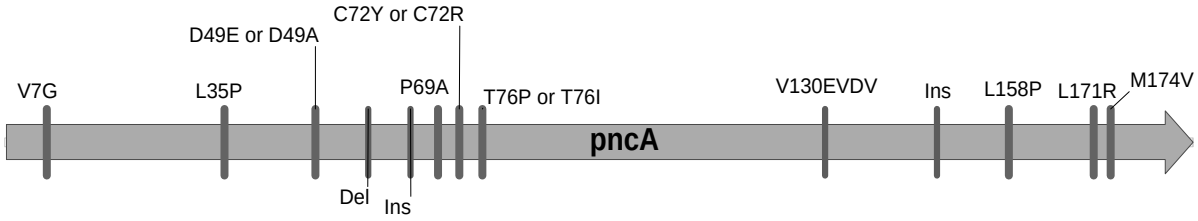


Fig. 36. Schematic representation of mutations observed in 561-nt *pncA* gene of *M. tuberculosis*. Ins and Del represents insertion and deletion respectively.

Point mutations were observed in 11 strains. All observed point mutations lead to an amino acid exchange. Insertions (TCCTCGTC, GAGGTCGAT and CG) were observed between position 192-93, 388-389 and 444-445 respectively in three different strains (Table 41

Table 41. The insert GAGGTCGAT and TCCTCGTC leads to tandem repeat. The first insert lead to repetition of AGGTCGATG or GAGGTCGAT twice (Fig. 37) and the second insertion lead to duplication of TCCTCGTC (Fig. 38)

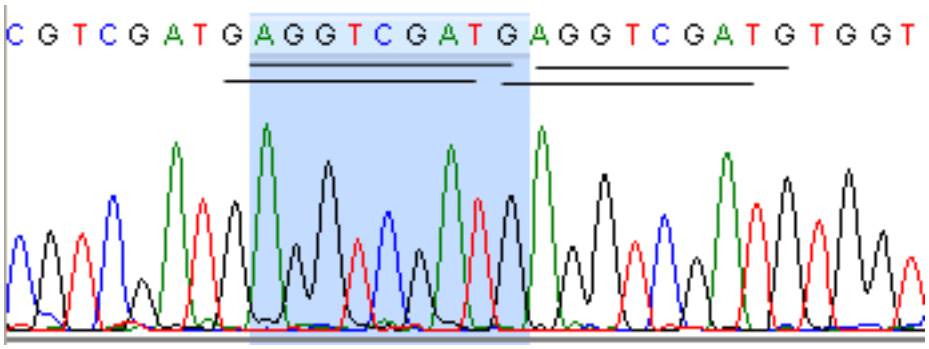


Fig. 37. Insertion of AGGTCGATG at 388-389 in *pncA* gene leading to tandem repeat of AGGTCGATG or GAGGTCGAT. The insertion is shown in grey and the tandem repeat is underlined.

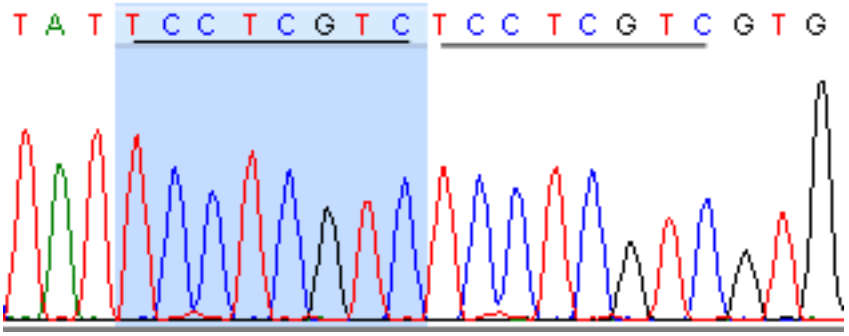


Fig. 38. Insertion of TCCTCGTC at 192-193 in *pncA* gene leading to tandem repeat of TCCTCGTC. The insertion is shown in grey and the tandem repeat is underlined.

Table 41. *pncA* mutation in *M. tuberculosis* isolates

Strain No.	Mutation in *pncA*	Amino acid level	PZA Susceptibility *	PZase Activity *	Mutation cited
103	20 T>G	V 7 G	R	-	This study
25	146 A>C	D 49 A	R	-	This study
37	146 A >C	D 49 A	S	+	This study
13	147 C>G; IS6110 at -30 nt	D 49 E	S	+	This study
47	146 A>C	D 49 A	R	-	This study
44	146 A >C	D 49 A	R	+	This study
92	104 T>G	L 35 R	R	-	This study; (Mphahlele et al. 2008)
33	172-173 Deletion of 2 nt	Frameshift	NA	NA	This study
26	192-193 TATCCTCGTC	Frameshift	R	-	This study
32	192-193 TATCCTCGTC	Frameshift	R	-	This study
101	192-193 TATCCTCGTC	Frameshift	R	-	This study
82	214 T>C	C 72 R	R	-	This study
41	215 G>A	C 72 Y	R	-	This study
107	226 A>C	T 76 P	R	-	This study; (Rodrigues Vde et al. 2005)
3	227 C>T	T 76 I	S	+	This study; (Park et al. 2001)
89	388 AGGTCGATG	V130EVDV	R	-	This study; (Rodrigues Vde et al. 2005)
40	444-445 Insertion of 2 nt	Frameshift	R	-	This study
20	457 C>G	L 158 P	R	-	This study
23	515 T>C	L 171 R	R	-	This study
66	523 A>G	M174V	R	-	This study

R: Resistance, S: Sensitive.

* PZA Susceptibility and whole cell PZase activity tests were done in clinical strains of *M. tuberculosis* and the data was kindly provided by Dr. M . Helena (Brazil).

In one strain, the IS-like element, IS6110 (X17348), was identified 31 nt upstream of the *pncA* gene. The sequence (1361 nt) contained the typical direct 3 nt repeats and inverted repeats (28 nt with 3 mismatched nt) at its extremities as described by Thierry (Thierry et al. 1990). This element disrupted Rv2044c, a gene for conserved hypothetical protein located downstream of Rv2045c (lipT). None of the analysed strains had mutation in *pncA* gene promoter region even 454 bp upstream of *pncA*.

Interestingly, none of the mutants reported in table 41 were detected in by Jureen (Jureen et al. 2008) in the strains studied in Stockholm, Sweden recently. Similarly, except one, none of the nucleotide changes detected in this study were reported by Jureen (Jureen et al. 2008) and Mphahlele (Mphahlele et al. 2008).

On comparing the mutation in the clinical strains of tuberculosis in *pncA* and *rpoB* genes, no clear association between these was be observed (Table 42) indicating that mutations in these genes arise independently.

Table 42. Mutations in rpoB and pncA

Strain No.	RIF*	PZA*	PZase*	Mutation *pncA*	Mutation *rpoB***
24	R	R	Pos	No Mutation	D184D, H526Y
37	R	R	Pos	D49A	F514 F, D516V
35	R	R		No Mutation	D516F
25	R	R	Neg	D49A	D516V
32	R	R	Neg	Frameshift	H526Y
33	R	R		Frameshift	S531L
73	R	R	Neg	No Mutation	D184D***
82	R	R	Neg	C72R	D184D***
87	S	R	Pos	No Mutation	D184D***
89	S	R	Neg	V130EVDV	D184D***
8	S	S	Pos	No Mutation	D184D***
27	S	S	Pos	No Mutation	D184D***
31	S	S	Pos	No Mutation	D184D***
57	S	S	Neg	No Mutation	D184D***
65	S	S	Pos	No Mutation	D184D***
78	S	S	Pos	No Mutation	D184D***

RIF: Rifampicin susceptibility, PZA: Pyrazinamide susceptibility, PZase: PZase Assay. R: Resistant, S: Sensitivity.

* Data provided from Dr. M.H Saad.

** The numbering used according to convention used for numbering *E. coli rpoB*.

*** Complete data of rpoB not available.

Previous published observation are further confirmed by the results presented in

Table 41 (see strains 25, 37, 44 and 47) which show that the whole cell mycobacterial PZAse assay does not correlate strongly to the standard antibiotic testing in media containing PZA. This assay further suffers from the reduced growth rate of *M. tuberculosis* under acidic environment.

We decided to take an alternative approach to solve this problem. We hypothesized that if the PncA (PZAse) of wild type and mutants could be cloned and expressed easily in *E.coli* followed by measurement of enzyme activity, it could be developed as a standard assay which even could be further improved for HTS use. The results described below were obtained from experiments performed with this objective.

3.2.2 Cloning, expression and purification of PncA

The complete genes *pncA* was amplified from *M. tuberculosis* H37Rv DNA using pncA-F2 and pncA-R2 primers (table 43, Fig. 39). The PCR products was cleaved and extracted which serve as DNA template for the next cloning primer. Cloning primers containing restriction sites (pncA-NdeI-F and pncA-Xho+SR or pncA-Xho-SR) were used for the re-amplification, which were used for the ligation in pET vectors (Novagen). Transformants in *E. coli* BL21 (DE3) were selected in LB agar with 50 µg/ml kanamycin. The positive transformants with insert were determined by checking the over expression of the protein of about 19.6 kDa in APS plate at 18°C for 3 days (Fig. 40 A). Nucleotide sequences of the insert in pET vector were determined by conventional DNA sequencing and the identification of the protein was confirmed by protein sequencing. The gene was cloned with and without histidine tag. Histidine tagged PncA was purified using a series of chromatography from Ni-NTA affinity chromatography to Q-Sepharose ion-exchange chromatography and finally with Sephadex-200 size exclusion chromatography (Fig. 40 B).

Table 43. PCR condition for the amplification of *pncA* (561 bp). Qiagen Master Mix or Bioline Master Mix was used for the amplification of *pncA*.

95°C	10 min	
95°C	30 sec	
57 °C	30 sec	30x
72°C	50 sec	
72°C	10 min	
4°C	forever	

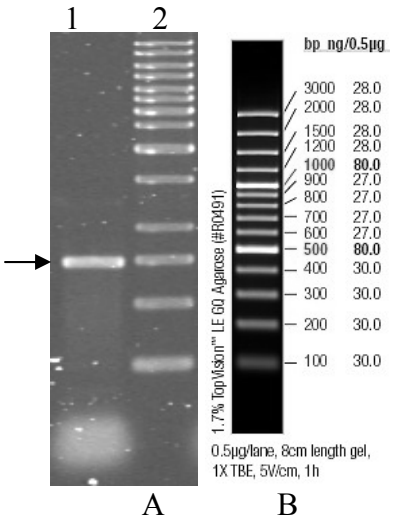


Fig. 39. 1% Agarose gel showing PCR amplified product of *pncA* from *M. tuberculosis* H37Rv. Lanes: 1, PCR product; 2, 100bp GeneRuler™ 100 bp DNA Ladder (Fermentas).

Fig. 39. GeneRuler™ 1kb DNA Ladder Plus (Fermentas).

Amplification of *pncA* from *M. tuberculosis* H37Rv DNA was done which was at the expected size of 561 bp as shown with an arrow.

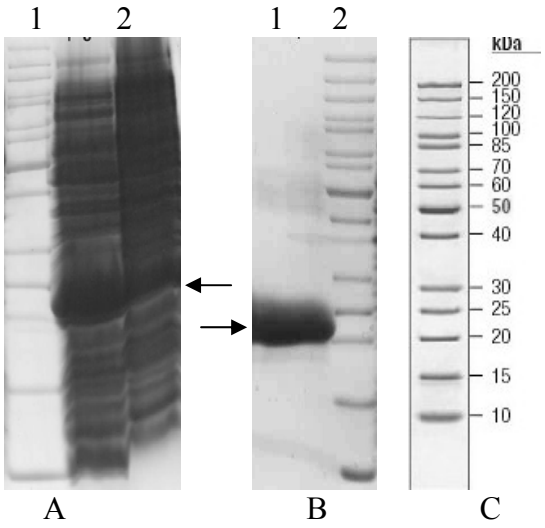


Fig. 40 A. SDS-PAGE of *M. tuberculosis* His-PncA (N-terminal His-tagged) expressed in *E. coli* BL21 (DE3) in APS medium at 18°C for 3 days. Lanes: 1, PageRuler™ Protein Ladder; 2, Soluble extract ; 3, Insoluble extract.

Fig. 40 B. SDS-Page of purified *M. tuberculosis* PncA from *E. coli* BL21 (DE3). Lanes: 1, Purified *M. tuberculosis* PncA; 2, PageRuler™ Protein Ladder (Fermentas).

Fig. 40 C. PageRuler™ Protein Ladder (Fermentas).

Similarly, amiD (another amidase) gene from *M. tuberculosis* H37Rv DNA was also amplified and cloned in *E.coli* BL21 (DE3) as shown below.

Table 44. PCR condition for the amplification of *amiD* (1428 bp). Qiagen Master Mix or Bioline Master Mix was used for the amplification of *amiD*.

95°C	10 min	
95°C	30 sec	
52°C	30 sec	30x
72°C	1 min 50s	
72°C	10 min	
4°C	forever	

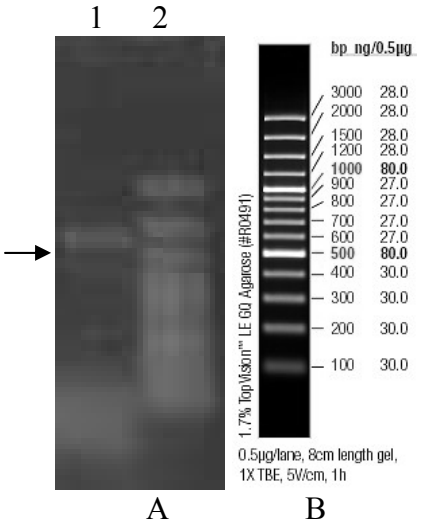


Fig. 41. 1% Agarose gel showing PCR amplified product of *amiD* from *M. tuberculosis* H37Rv. Lanes: 1, PCR product; 2, 1Kb GeneRuler™ DNA Ladder (Fermentas).

41 B. 1Kb GeneRuler™ DNA Ladder (Fermentas).

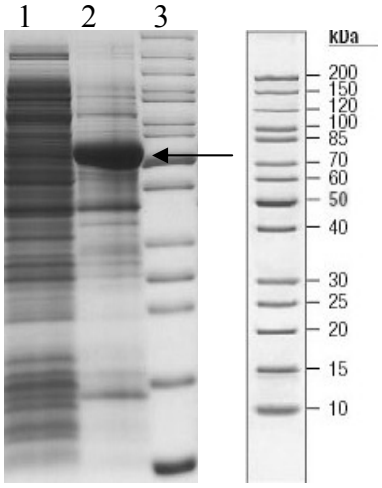


Fig. 42 A. SDS-PAGE of *M. tuberculosis* amiD expressed in *E. coli* BL21 (DE3) in APS medium at 18°C for 3 days. Lanes: 1, Soluble extract; 2, Insoluble extract; 3, PageRuler™ Protein Ladder.

42 B. SDS-Page of purified *M. tuberculosis* PncA from *E. coli* BL21 (DE3). Lanes: 1, Purified *M. tuberculosis* PncA; 2, PageRuler™ Protein Ladder (Fermentas).

3.2.3 Cell free PZase assay

Direct enzymatic assay of PncA was developed based on Allen's (Allen et al. 1953) description of formation of orange red complex during the reaction of POA with ammonium ferrous sulphate. This assay was tested with purified PncA enzyme.

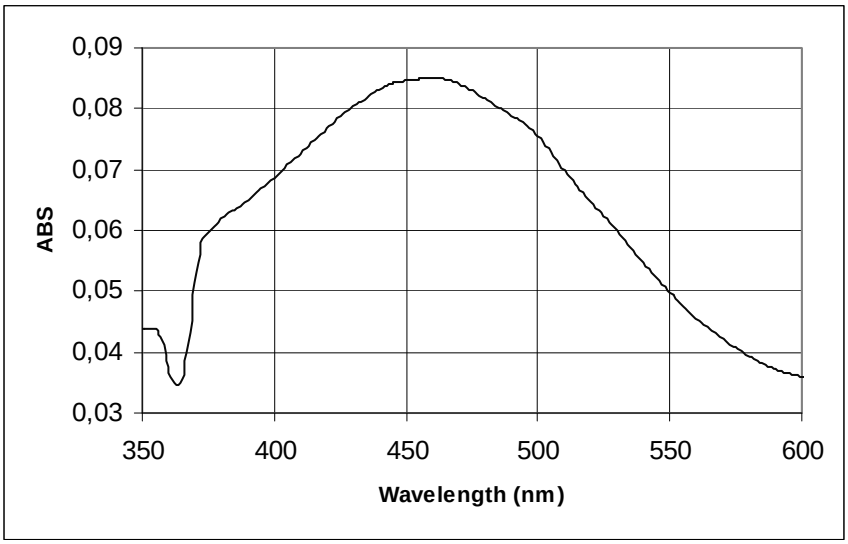


Fig. 43. Absorption spectra of complex of POA and Mohr's Salt.

The characterization of pyrazinamidase was done by measuring the absorbance of coloured complex formed from the product (POA) and Mohr's salt at 450 nm. The initial velocity of the reaction was calculated with the initial slope (OD_{450}/min) and the extinction coefficient of the POA and Mohr's salt. These rates were applied for the kinetic parameter with GraphPad Prism v4.0.

PncA reaction with 500 µM of PZA and 2 µg of PncA at 100 mM Glycine pH 6.0 was used for the calculation of the specific activity of PncA. A standard curve was prepared by treating different known concentrations of POA and Mohr's salt in 100 mM Glycine buffer pH 6.0.OD_{450} measured during the kinetics of the assay after 2 min and 4 min of addition of PncA were converted to equivalents of POA by referring to the standard curve. It was observed that the specific activity of purified PncA was 10-11 U/µM. 1 U of pyrazinamidase was defined as the amount of PncA that produced 1 µM of POA per minute.

Table 45. Enzymatic characterization of the PncA

Michaelis-Menten	
Best-fit values	
Vmax	357
K_m	232
Std. Error	
Vmax	21
K_m	34
95% Confidence Intervals	
V_{max}	312 to 401
K_m	159 to 305
Goodness of Fit	
Degrees of Freedom	16
R^2	0.9637
Absolute Sum of Squares	5199

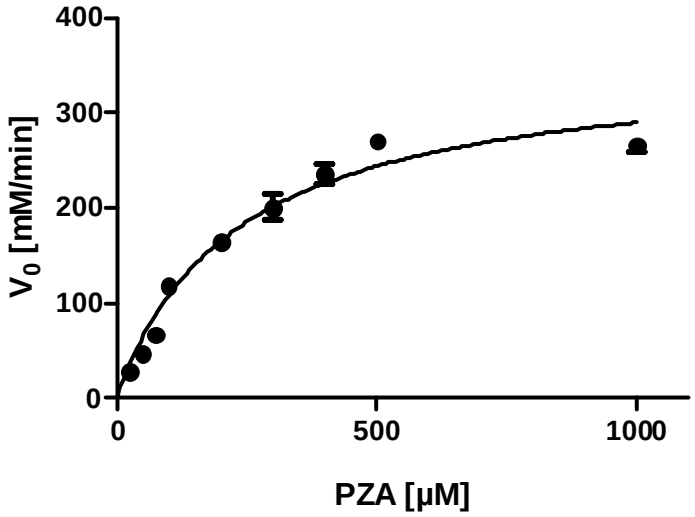


Fig. 44. Michaelis-Menten plot. Reaction rate (V_0) plotted against PZA concentration.

The K_m value for PZA 232 µM was comparable with other 300 µM (Boshoff and Mizrahi 1998). The high value shows a low specificity to the substrate.

Effect of pH

The effect of pH in the enzyme assay was limited with this assay. An increment of the rate of reaction up to pH 6.2 was observed, however after 6.4 there was unusual character. It shifted down suddenly at this pH and tend to increase till pH 7.2 (Fig. 46). This could not be attributed only to the enzyme. Measurement of activity above pH 7.2 was limited due to the precipitation of Mohr's salt at this pH.

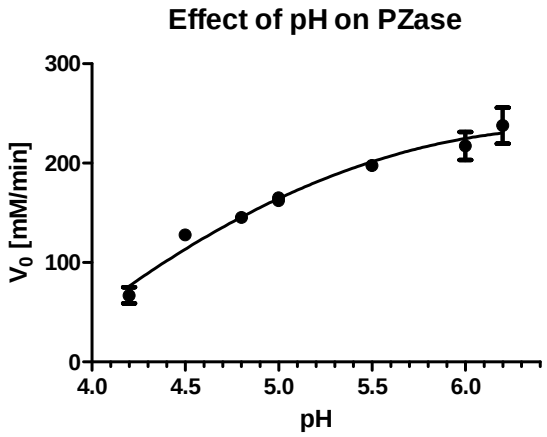


Fig. 45. Effect of pH on *M. tuberculosis* PncA. 100mM Glycine Buffers of different pH were used for the measurement.

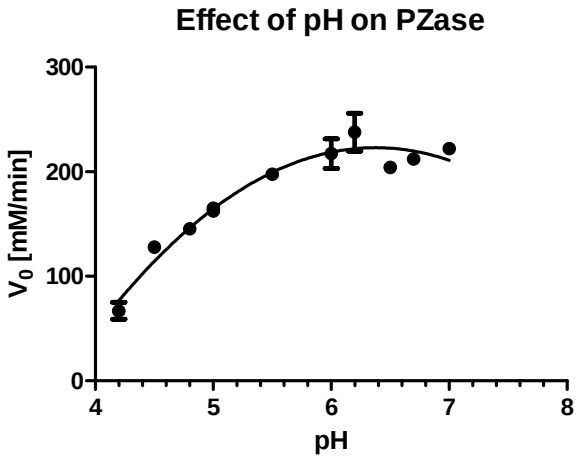


Fig. 46. Abnormal character of PncA enzyme activity between pH 6.4 till pH 7.2. The pH characterization beyond pH 7.2 was not possible due to the precipitation of Mohr's Salt.

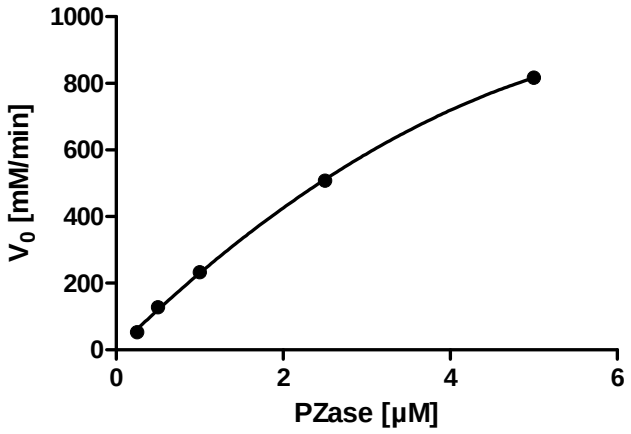


Fig. 47. The increase of rate of reaction with increasing concentration of PncA.

Thus, we could successfully establish an enzyme assay with the recombinant mycobacterial PZAse purified from from *E.coli.* In the next step similar attempts were made a series of pncA mutants.

3.2.4 Cloning and expression of mutant PZase

The DNA from clinical strains of *M. tuberculosis* containing mutation in *pncA* were used for the amplification of *pncA* gene with primers pncA-NdeI-F and pncA-Xho-SR. The DNA after extraction were cloned in *E. coli* BL21 (DE3) using pET-26b(+) and or pET-28b(+) vectors. The selection of the transformants was done in LB agar with 50 µg/ml kanamycin. The positive clones were selected by checking the over expression of protein of about 19.6 kDa in APS agar with 50 µg/ml kanamycin. 96 well plate DNA preparation (2.6.1.3) was done from the positive clones and the plasmid extracted were sequenced for the confirmation of the clones and mutation, thus identifying the *pncA* mutants.

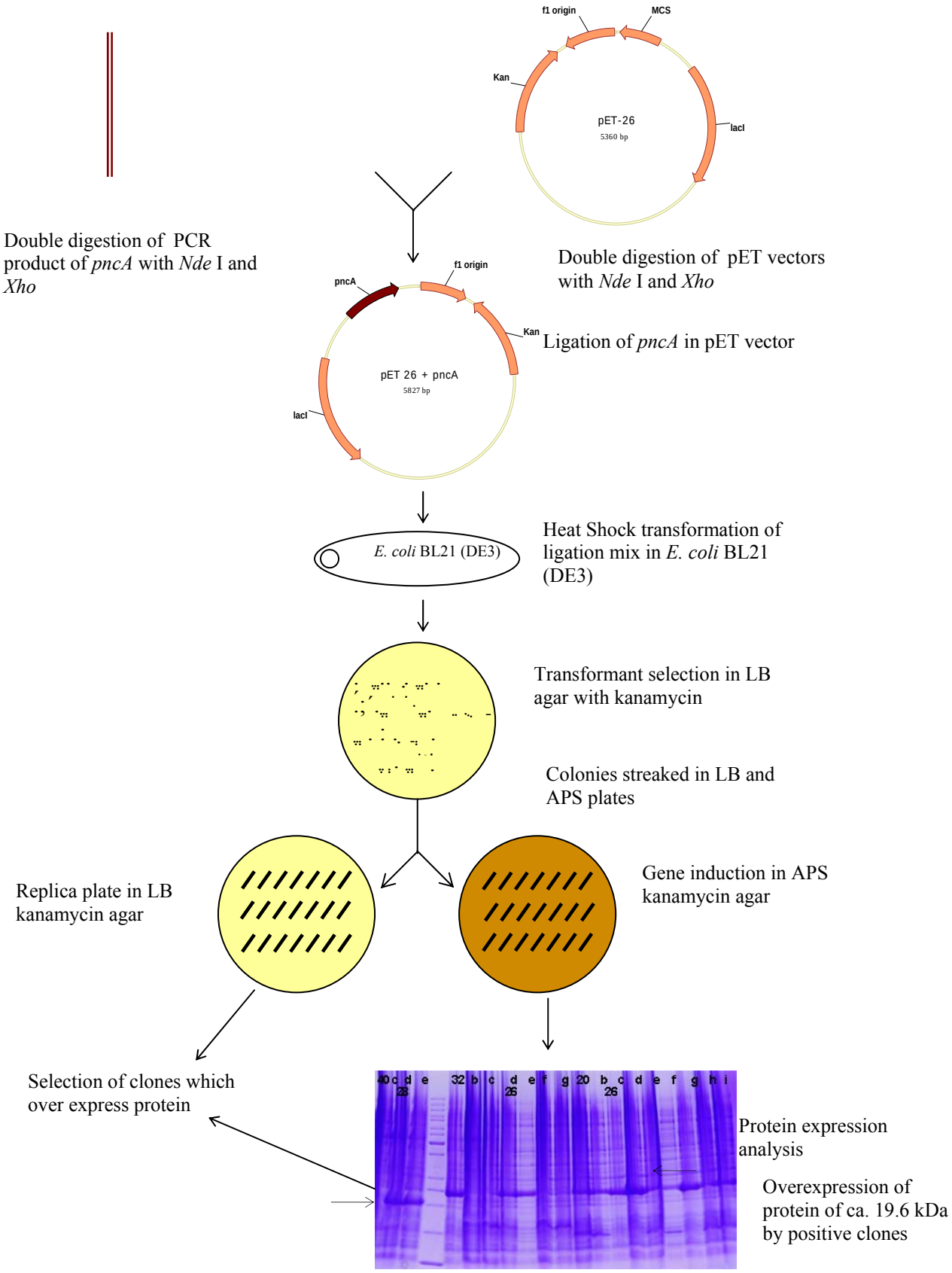


Fig. 48. Strategy used for the cloning of *pncA* mutants.

3.2.4.1 Cell free PZase assay with soluble lysate of the *pncA* mutants

The recombinant *pncA* mutant clones were cultured in APS agar with 50 µg/ml kanamycin in APS autoinduction medium. The bacteria were then suspended in either a 96 well plate or in a 96 deep well plate. The cells in 96 well plate were lysed with Bugbuster, while the cell in 96 deep well plate was disrupted using ultrasonication.

Supernatant fraction was separated from the lysate after centrifugation (3000 x g; 15 min at 4°C). The protein concentration of the supernatant was determined. 10 µg of soluble cell extract was pipetted with Biomek 2000 in the micro plate containing 50 µl of 100mM Glycin pH 6.0; to which 50 µl of the mixture containing 1 mM PZA and 2 mM ammonium ferrous sulphate 100 mM Glycine. HCl pH 6.0 was pipetted. The reaction was instantly measured with Dynex Microplate reader continuously at an interval of 20 sec for 20 minutes.

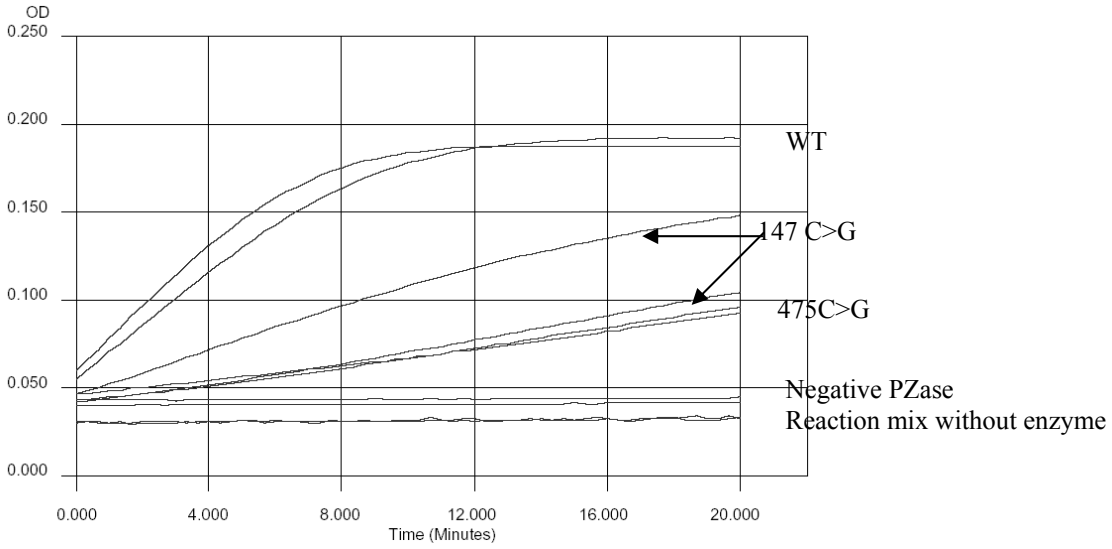


Fig. 49. Result from high through output assay of cell free PZase assay. 10µg of cell extract of wild type (WT) and various mutants in the PncA reaction mix. Two of the mutants had deficient PncA activity.

The result from Fig. 49 show that mutations 147 C>G and 475 C>G lead a partial reduction in enzyme activity. Thus, by this method quantitative results for pncA (PZAse) of a mycobacterial strain could be obtained within 2-3 days as compared to several weeks by currently standard methods.

Interestingly, the colour formation in microtiter wells could easily differentiate between positive and negative PZAse results (Fig. 50).

Fig. 50. Microtiter plate wells showing the cell free PZase assay with pncA mutants (upper wells) and controls (lower wells).

3.2.5 Development of a whole cell PZase assay with the recombinant *pncA* mutants

Following the highly encouraging results obtained with the recombinant pncA clones in the cell free assay, we then attempted to develop an even more rapid and direct test for measuring pncA in recombinant *E.coli* strains.

A preculture of *pncA* mutants in *E. coli* BL21 (DE3) was prepared from the overnight cultured clones in LB Medium with kanamycin. 100 µl from the overnight culture was used to inoculate 2.5 ml LB-medium pH 5.5 with 50 µg/ml kanamycin and 100 µg/ml PZA ml in a test tube. Alternatively 4 µl of the innoculum was used to inoculate 100 µl of LB-medium pH 5.5 with 50 µg/ml kanamycin and 100 µg/ml PZA ml in a 96 well plate. The initial OD_{600} in both case was approximately 0.02. Incubation was followed at 37°C for 3 hrs. After 3 hrs, 0.5 ml of 1% solution was added in each tube containing 2.5 ml culture and 40 µl of 1% ammonium ferrous sulphate solution was added in the 96 well plate containing 100 µl of culture. With this assay, orange-red colouration was developed immediately after the addition of ammonium ferrous sulphate because the

POA produced by the cells is excreted into the medium. The PZase negatives could be clearly identified because there was no change in colour with the addition of ammonium ferrous sulphate (Fig. 50). Assay done in test-tube with 2.5 ml of the culture media and 200 µl in 96 microtiter plates were identical. As expected the colouration produced in various mutant clones were of variable intensity showing variable degree of attenuation of the PZAse in different mutants (Fig. 50).

Fig. 51. Whole cell PZase assay in liquid cultures. Media: medium only; *pncA* Mutant: contains insert with mutation in *pncA* gene; *PncA* clone and pncA clone 2 contain *pncA* wild type gene; *amiD* contains *amiD*; Empty clone contains only pET-26b(+) vector without any insert. The host strain was *E. coli* BL21 (DE3) in all cases. The orange-red were PZase Positive. A mutant was slightly positive and Empty clone and *amiD* clone were PZase negative. Media was used for the control.

A comparison of results obtained with the the PZA resistant and sensitive strains from Brazil using the standard mycobacterial PZAse assay and the new methods developed in this study are presented in table 46. In addition, the results confirm that amid gene product of M. tuberculosis is unable to use PZA as substrate. Thus, as in *M. bovis* and *M.bovis* BCG, pncA is the only functional gene in *M. tuberculosis* coding for a functional PZAse. All strains of *M.bovis* reported till today are resistant to PZA due one single point mutation which completely inactivates the enzyme.

Table 46. Comparison of standard mycobacterial PZAse with the new methods developed in this study.

Mutation in pncA	Amino acid level	Change it to OD after 10 min	PZase Assay with pncA Mutants *	Cell free PZase assay with Soluble Fraction	Mycobacterial PZase Assay **
35A>G, 240C>T	D 13 G, D 80 D	0,067	0	Negative	
100 T>C	Y 34 H	0,044	0	Negative	
147 C>G	D 49 E	0,108	4	Slightly positive	Pos
172-173 Deletion2bp	Frameshift	0,04	0	Negative	NA
188 A>G	D 63 G	0,044	0	Negative	
192-193 Insertion 8bp	Frameshift	0,056	0	Negative	Neg
194 C>T, 241 T>A	S 65 F, F81 I	0,055	0	Negative	
215 G>A, 515 T>C	C 72 Y	0,032	0	Negative	Neg
226 A>C	T 76 P	0,038	2	Negative	Neg
272A>G, 353A>G,515T>C	E 91 G, N 118 S, L 171 P	0,043	0	Negative	
281T>C, 515T>C	F 94 S,L 171 P	0,03	0	Negative	
287 A>G	K 96 G	0,048	0	Negative	
294C>T, 515C>T	A 98 A, L171 P	0,044	0	Negative	
350T>C	L 117 P	0,035	4	Positive	
355T>C	W 119 R	0,059	0	Negative	
372-373 Insertion 9bp	Frameshift	0,047	0	Negative	Neg
392T>A	V 131 D	0,036	0	Negative	NA
444-445 Insertion 2bp	Frameshift	0,045	0	Negative	Neg
475C>G	L 158 P	0,051	1	Negative	Neg
507C>T	V 168 V	0,055 §	5	Negative??	
Without any insert	NA	0,072	0	Negative	-
With amiD	NA	0,03	0	Negative	-
With other gene	NA		0	Negative	-
Wild Type	NA	0,184	5	Positive	Pos
E.coli BL21 (DE3)	NA	0,032	0	Negative	

* Extent of colour production after addition of 1% ammonium ferrous sulphate. 0: No colour produced, 1 to 5 increasing colour production.

** Data provided from Dr. M.H Saad.

§ Contains less amount of protein (Data not shown).

The data presented in table 46 shows that the PZA assays developed in this study allow rapid decision about the PZA resistant or sensitive phenotype as compared to the conventional mycobacterial tests where it is often difficult to interpret the results. Only the 507C>T mutation gave unexpected negative result but on examination of the SDS gel it was noticed that the extract for this clone did not contain sufficient amount of recombinant protein.

3.2.6 Structural analysis of PZase

The objective of the results described in this section was to find out whether the data obtained in this study on PZA resistant and sensitive strains from Brazil show a correlation between functional and structural features of pncA (PZAse).

The *M. tuberculosis* PncA was subjected to a BLAST analysis against the Swiss-Prot/TrEMBL database. (http://www.expasy.org/tools/blast/). The sequences were used for a further PRATT analysis to identify a conserved pattern within the achieved sequences.

The pattern D-x-Q-x-[DT]-F-x(2)-[DGS]-[GS]-x-[AGLV]-[AGPST]-x(4)-[ADEGNQS] was identified. This pattern was found to be typical for the PncA protein family. Typical members of this family are PNC1 of *Saccharomyces cerevisiae* (P53184) and Pyrazinamidase/nicotinamidase of *E. coli* (P21369).

With Clustal alignment 10 absolutely conserved amino acids were identified among the PncA family. The result agree with the findings of Du (Du et al. 2001). The identified conserved residues match well with the active site residues for *P. horikoshii* pyrazinamidase. Du et al. proposed a catalytic mechanism, involving Lys94, Cys133 and identified a zinc ion, crucial for catalysis.

Table 47. Totally conserved residues among the pncA family.

M. tuberculosis	*S. cerevisiae*	*P. horikoshii*
Asp8	Asp8	Asp10
Gln10	Gln10	Gln12
Asp12	Asp12	Asp14
His51	His53	His54
His71	His94	His71
Lys96	Lys122	Lys94
S104	S132	S104
Leu120	Leu149	Leu115
Gly132	Gly161	Gly127
Ala134	Ala163	Ala129
Cys138*	Cys167*	Cys133*

* This AA is not completely conserved among all pncA proteins

Modelling was done with the Swiss Model server using the default parameters for the first approach mode. The model computed is based on the template of the yeast nicotinamidase Pnc1p (PDB ID: 2h0r)

The model aligned very well with the *P. horikoshii* and *S. cerevisiae* nicotinamidase. The root square deviation, which measures the average distance between the backbones of superimposed proteins is 0.6 and 0.5 respectively. All residues of the active site of *M. tuberculosis* PncA, *S. cerevisiae* nicotinamidase and *P. horikoshii* enzyme are completely conserved and aligned completely.

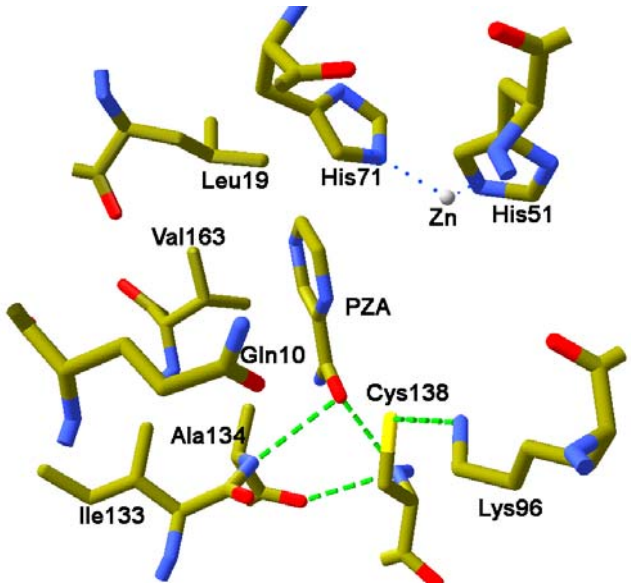


Fig. 52. Model of PZA located in the active site of PZase of *M. tuberculosis*.

A model of pyrazinamide was used to model the position of pyrazinamide in the active site (Fig. 52). PZA was positioned according the catalytic model of Du (Du et al. 2001). The oxygen of the carboxamide group was positioned between the amide groups of the main chain of Ala134 and Cys138 to form hydrogen bonds. The final position of the oxygen was modelled at the position of water 297 in the 2h0r. In the final model the oxygen of the carboxamide group forms hydrogen bonds to the main chain of Ala134 and Cys138. No clashes between the PZA and the protein residues occur.

Based on this model one can conclude that the active site is formed by Leu19 and Val163 to form a hydrophobic pocket for the PZA ring. Backbone amides of Ala134 and Cys138 bind the oxygen of PZA. His71 and His51 bind Zinc, which has a crucial role in catalysis Du (Du et al. 2001).

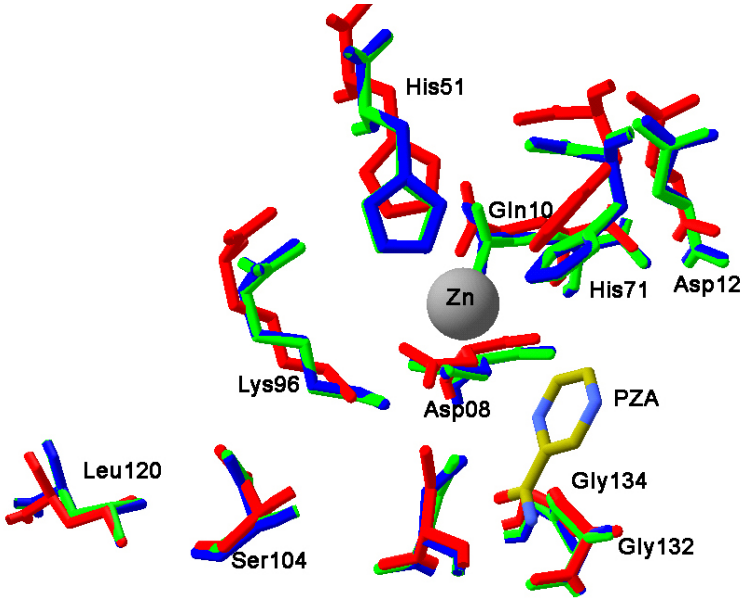


Fig. 53. Structural alignment of amidases from *P. horikoshii* (Red, PDB ID: 1IM5), *M. tuberculosis* (Green, Theoretical model) and *S. cerevisiae* (Blue, PDB ID: 2h0r). The amino acid residues number convention followed corresponds to *M. tuberculosis* PZase.

The catalytically important residues Cys138 and Ala134 act via main chain bonds. Therefore, a substitution with an amino acid of the same size may not be detrimental for catalytic activity. His71 and His51 appear to be the most important residues, due to their role in zinc binding.

The analysis of PncA mutants presented in this study correlates well with the model discussed.

4 Discussion

The two main problems concerning TB therapy today are,

1. Long-term treatment which often leads to patient non-compliance resulting in the emergence of drug resistant TB which frequently is untreatable with current drugs,

2. There are no drugs to treat persistent/dormant and XDR TB

Rifampicin and Pyrazinamide are two highly useful first-line TB drugs with antibiotic activity against replicating and dormant *M. tuberculosis*, respectively. Unfortunately resistance to these antibiotics is quite frequent. This problem of drug resistance can only be solved by intensive efforts in drug development which mostly rely on efficient assay system suitable for HTS of drug candidates. Current assays for RNAP (Rifampicin target) and PZAse (Pyrazinamide target) are unsuitable for such use.

Here we present two novel strategies for assaying RNAP and Pzase with a potential application in HTS for drug development against *M. tuberculosis* and for the diagnosis of Rifampicin- and/or PZA-resistant clinical strains of *M. tuberculosis.*

4.1 RNAP

Bacterial DNA-dependent RNA polymerase is an attractive drug target because RNA chain elongation is essential for bacterial growth (Jin and Zhou 1996). RNAP Assay can be used for screening chemicals which may lead to a hit for RNAP.

The current assays for the activity of RNAP require the use of radiolabeled (Daniel et al. 1975; McClure 1980; Wu et al. 1997) or chemically modified nucleotides like fluorescent derivative of nucleotide (Bertrand-Burggraf et al. 1984; Kozlov et al. 2005; Bhat et al. 2006) or other derivative of nucleotide (Vassiliou et al. 2000) or detection of end product of transcription like RNA (Kuhlman et al. 2004) or PPi (Johnson et al. 1968). These assays to monitor RNAP activity depend on the detection of radiolabeled RNA product synthesized during the transcription process. Most of the assays are gel-

based in which radiolabeled RNA is visualized in gel electrophoresis or use scintillation counter. Although radioactive signals are easily detected and quantified, these methods have disadvantages such as user exposure to radiation, short shelf life, high handling cost for radioisotopes and high labour costs. The use of modified florescent nucleotides, on the other hand has not been successful as a routine assay.

For a HTS, the use of live *M. tuberculosis* or strain extracts are not appropriate, not only because it is an highly infectious organism, but also because it is an extremely slow growing bacterium. Thus, for an HTS of drugs or drug candidates as inhibitors of RNAP of *M. tuberculosis,* there is an urgent need for a rapid and feasible assay.

The purification of large quantity of native RNAP from *M. tuberculosis* is very labour intensive, hazardous and time consuming and cumbersome process. In an effort to improve the characterization of polymerization and develop new antibiotics that target RNAP, a nonradioactive assay was developed, which does not utilize radioactive or florescent nucleotides but can use natural nucleotides. A coupled-enzyme system based on *E. coli* RNAP and the luminescent detection of ATP by converting PPi using ATP sulfurylase through successive steps was devised. The background of the reaction was lowered by using apyrase to degrade the residual nucleotides after the RNAP reaction prior to converting PPi to ATP.

Although the RNAP Assay discussed is simpler than the classical radioactive test, also suffers from some draw backs as other test due to the multiple steps it has to pass through. In every step there is a danger that one of the other enzyme than RNAP may be affected by the substance tested which may or may not wholly affect the result, thus it might be necessary to include the substance as a control in case a hit is sought out. On the other hands, the light produced from luciferin and luciferase system varies according to the batch and the storage of the detection system. However this draw back can be tackled by using the controls every time and analysing the result in terms of controls.

The other crucial process in the RNAP assay is the effect of temperature gradient, when incubating 96 well plate. This was solved by using a thermocycler for the purpose of incubation and denaturation of the enzyme. The measurement however should be carried out in a white plate.

The new assay developed in this work is simple, cost effective and easy to handle. Also the reagents required for the assay are all commercially available.

One of my objectives was to replace *E. coli* RNAP with *M. tuberculosis* RNAP remained unfilled. RNAP is a huge complex, which require at least three components viz. RpoB, RpoC and two RpoA to form functional enzyme. During the course of work, several methodologies were attempted to produce functional *M. tuberculosis* RNAP, however a functional RNAP as expected could not be produced, although other (Jacques et al. 2006) have shown to produce functional one. It is easy to understand that even if one of the component do not orient properly during the composition of the complex, it will not work.

4.2 Pyrazinamidase

The mechanism by which the PZA functions in the TB remains still incomprehensive though vast amount of research has been done. The PncA in *M. tuberculosis* is deemed to be responsible for the conversion of PZA to POA, which in turn acts in the Mycobacteria to kill the cells. The inhibition with POA in mycobacteria is still illusive whether it is due to general intercytoplamic acidification or due to inhibition of specific cellular target. The physiological role of the nicotinamidase in most prokaryotes is to degrade nicotinamide to nicotinic acid, which in turn is recycled to NAD via the Preiss-Handler pathway of the pyridine nucleotide cycle (Foster and Moat 1980).

Besides *pncA*, there are several other putative amidases like *amiA1*, *amiA2*, *amiB1*, *amiB2*, *amiC* and *amiD*. Concerning the resistance developed by *M. tuberculosis* BCG against PZA and the probable amidases present in both the *M. tuberculosis* BCG and *M. tuberculosis* H37Rv, alignment of the protein sequences of all these amidases show that only *pncA* contains mutation at 169 C>G (His57Asp) which can be accounted for the resistance it develops against PZA. During the sequencing of *amiD* gene from several strains, a novel mutation at 220 G>T (Ala74Ser) was encountered, however due to its presence in both PZase positive and PZase negative as well as in both PZA sensitive and PZA resistant strains. The *M. tuberculosis* AmiD was also not able to take PZA as a substrate.

A modification of Wayne's method of determining the PZase activity of strains was performed in *E.coli* BL21 (DE3) mutants of *pncA*. In this assay the activity of the mutant could be accessed directly. PZA converted into POA was secreted in medium, which then react with ammonium ferrous sulphate to produce a red-orange complex. The same test with *amiD* was negative, conferring the inability of *amiD* to act on PZA.

The excretion of amidase by *M. tuberculosis* in culture filtrate is already known (Raynaud et al. 1998). The resistance developed by PZase positive organism like *M. smegmatis* may be due to the location of the enzyme. In *M. tuberculosis* the enzyme is found extracellularly in the culture filtrate while in *M. smegmatis* it is located deep inside the cell envelope (Raynaud et al. 1998), which can be accessed only after treating the cells with Tween 80 for 24 hours and glass beads.

PZA susceptibility testing is difficult and often unreliable because of the acid pH requirement for drug activity (Hewlett et al. 1995). The problem of the susceptibility testing is the inhibition by acid such that at least 10% of clinical isolates cannot be tested because they fail to grow on the acid pH medium (pH 5.5) (Siddiqi 1992). The most common problem is false resistance which is caused by large inoculum of a sensitive strain (Hewlett et al. 1995). In our studies also we have encountered incompatibility of the data in the mutation (D49A) observed and the PZA susceptibility test. PZA susceptibility has also been determined by detecting PZase activity of cultured *M. tuberculosis* (McClatchy et al. 1981; Miller et al. 1996; Suzuki et al. 2002) since PZase activity is lost during PZA resistant isolates (Konno et al. 1959; Zhang and Mitchison 2003). However, the interpretation of classical PZase assay done according to Wayne's method lacks a clear interpretation. This is due to the instability of ammonium ferrous sulphate, which is the core reagent used for the development of the colour. At one hand it produces brown colour in alkaline pH at the other hand, the reagent itself is not stable for a long period of time. It slowly develops brown colouration with time even at 4°C within a week and at RT the reagent develops brown colouration within a few days. The clear interpretation of Wayne's method is thus difficult because the Mycobacteria takes time to grow and the reagent to access the assay is not stable. At pH higher than 7.0, the reagent auto precipitates, thus rendering it unusable as a reagent at alkaline condition.

The *M. tuberculosis pnc*A gene was cloned, over-expressed, purified to homogeneity and enzymatic characterization was done. It was also observed that the enzyme was able to take both PZA and nicotinamide (data not presented). The enzyme was found to have an activity of 10-11 U/μM. The exclusion of PncA during the purification of size exclusion chromatography showed it to be a monomer.

Identification of pncA mutants involved in PZA resistance: *M. tuberculosis* PZase enzyme is encoded by the *pncA* gene (Scorpio and Zhang 1996). Mutation in the gene causing PZA resistant have been well characterized (Scorpio et al. 1997; Lemaitre et al. 1999; Mestdagh et al. 1999; Cheng et al. 2000; Morlock et al. 2000; Huang et al. 2003; Portugal et al. 2004; Jureen et al. 2008) and are located along the whole gene as well as in the upstreamed putative regulatory region.

Mutation in *pncA* may be responsible for the conformational change which may affect enzyme activity. While the mutation in putative regulatory region of the gene may hinders RNA polymerase to bind to the promoter, thus disturbing the active transcription. There are also reports regarding PZA resistant strains that maintain PZase activity (Cheng et al. 2000; Lee et al. 2001), this has lead to speculation of other mechanism than the PZase activity. However, partial inactivation of the pyrazinamidase may also lead wrong interpretation of the activity, an example of partial inactive PncA activity was encountered which is shown in Fig. 49. PZase activity affects the rate of conversion of PZA to bactericidal POA, which would therefore be an important factor in determining the susceptibility of mycobacteria to PZA, this was also observed by Sun (Sun and Zhang 1999). Defective PZase activity caused by pncA mutations were also correlated with PZA resistance in *M. tuberculosis* (Scorpio and Zhang 1996; Scorpio et al. 1997; Sreevatsan et al. 1997; Zhang et al. 2008).

PZA resistant strains must not always be PZase positive (Butler and Kilburn 1983) and thus do not necessarily always contains mutation in *pncA* gene. The same we have also encountered during the sequence analysis of the clinical strains of tuberculosis. This suggest for an alternative mechanism of the resistant organism to tackle with PZA. Pyrazinamidase however does have influence in the susceptibility as *pzaA* or *pncA* from *M. smegmatis* when introduced in to naturally PZA resistant *M. tuberculosis* BCG, the resistant *M. tuberculosis* BCG became susceptible to PZA (Guo et al. 2000). Similarly,

Sun (Sun and Zhang 1999) reported partial PZA susceptibility in *M. bovis* BCG when it was transformed with *pncA* gene from *M. kansasii*. *M. kansasii* is also PZA resistant but having reduced PZase activity. On introducing *pncA* gene from *M. avium*, the organism became highly susceptible to PZA (Sun and Zhang 1999). Thus showing the direct relationship between PZase and PZA.

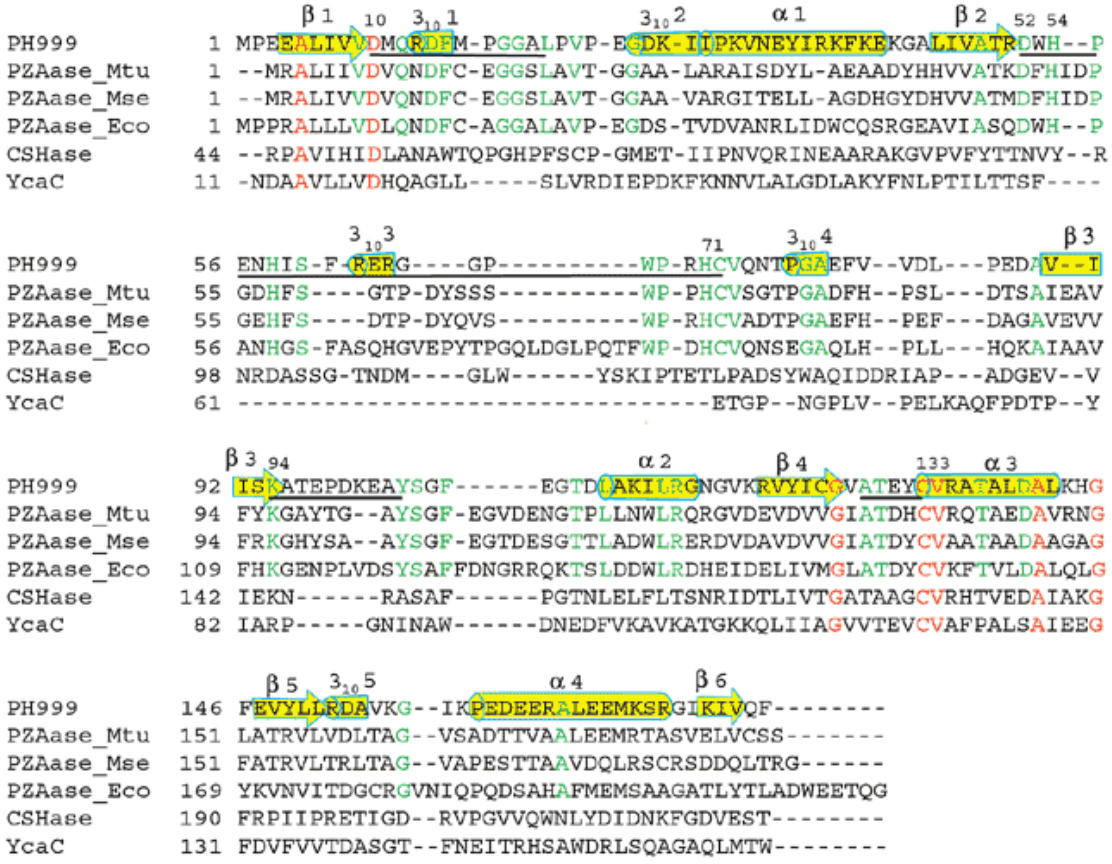


Fig. 54. Multiple sequence alignment of nicotinamidases of *P. horikoshii* (PH999) (BAA30096), *M. tuberculosis* (PZase_Mtu) (AAB37768), *M. smegmatis* (AAD11442), *E. coli (P*, and, *N*-carbamoylsarcosine amidohydrolase (CSHase) (P32400) of *Arthrobacter sp*, and YcaC (P21367) of *E. coli* (P21367). The residues conserved in the nicotinamidases are coloured in green. The residues conserved in all six sequences are coloured in red. The residues in the four loops that line the active site are underlined.(Du et al. 2001)

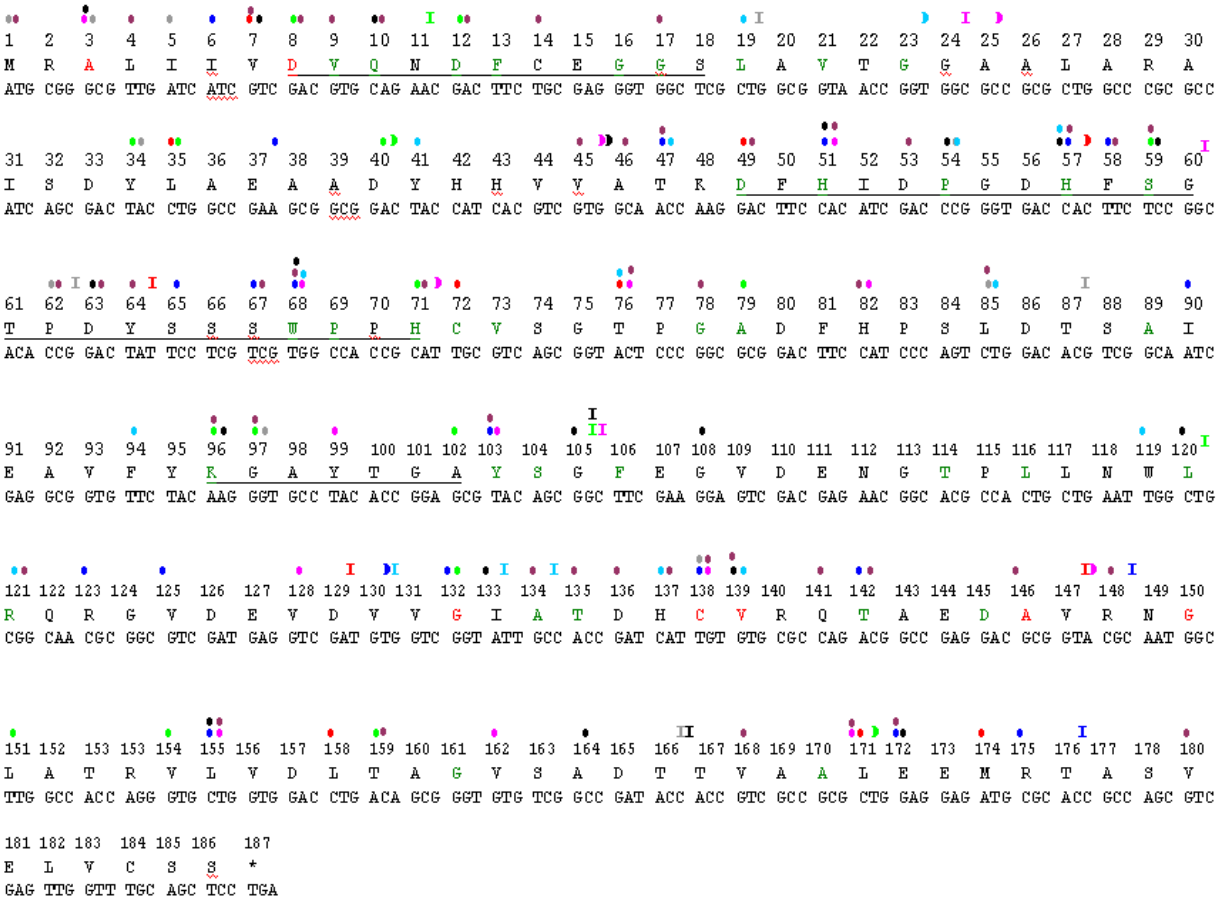


Fig. 55. Overview of mutations present in the pncA gene of M. tuberculosis. The residues conserved in the nicotinamidases are coloured in green. The residues conserved in all six sequences are coloured in red. The residues in the four loops that line the active site are underlined.(Du et al. 2001). Red: This study; Green: Studies from South Africa (Mphahlele et al. 2008); Blue: Studies from Sweden (Jureen et al. 2008); Black: Studies from Brazil (Rodrigues Vde et al. 2005); Green: Studies from Japan (Miyagi et al. 2004); Studies from southeast Brazil (Barco et al. 2006); Light Blue: TB from diverse geographic localities (Sreevatsan et al. 1997); Purple: Analysis of published literatures from different part of world (O'Sullivan et al. 2005). o : Point Mutation; I: Insertion, D : Deletion.

The mutations in *pncA* were distributed equally over the whole gene (Fig. 36 and Fig. 55) as in the previous studies (Scorpio et al. 1997; O'Sullivan et al. 2005; Rodrigues Vde et al. 2005; Barco et al. 2006; Jureen et al. 2008). The new mutations are being continuously reported in new studies. In a recently published paper (Mphahlele et al. 2008) a new mutation common to our studies was published. There has been 197 different mutations reported in the gene and its promoter region. It can thus be expected that more new mutation could be detected in the gene. The reason for the occurrence of wide variety of mutation in the gene is not known, it was only believed that the *pncA*

gene is located in a hotspot for mutation in the mycobacterial genome (Barco et al. 2006).

It is generally considered that mutations which lead to PZA resistance are distributed randomly along the gene, however some authors mentioned a certain degree of conservation of *pncA* mutations at amino acid residues 3-17, 61-76 and 132-142 in PncA protein (Scorpio et al. 1997; Lemaitre et al. 1999; Park et al. 2001). The crystal structure of PZase of *P. horikoshii* shows that the resides 10 to 21, 128 to 133, and 52 to 72 contribute most of the scaffold of the active site (Du et al. 2001). However revision of mutations in the gene (Fig. 55) shows that mutation in *pncA* gene are random and throughout the gene without showing any pattern.

Another interesting feature found was duplicated nucleotide sequence in *pncA* gene in some strains. The duplicated sequence could have been remnants of a mobile element which was integrated between the repeats. During the course of sequence analysis, it was observed that two types of different insertions ocurr which lead to tandem repeat of 8 bp or 9 bp twice. In one of the sequence, where 9 bp (AGGTCGATG) are added between 388-389 bp from the start codon, tandem repeat of AGGTCGATG or GAGGTCGAT was observed (Fig. 37). Although Rodrigues (Rodrigues Vde et al. 2005) also found the same insertion, they did not report it as repeat. Similarly, insertion of TCCTCGTC between 192-193 in *pncA* gene leads to two tandem repeat of TCCTCGTC (

Fig. 38). This was observed in three strains. This type of tandem repeat could be the remains of an IS element. Transposition of IS1660 inside the *pncA* gene was already described by Lemaitre (Lemaitre et al. 1999). With the known duplication only it was not possible to find the nature of the mobile element which was stationed previously.

In addition, the results confirm that amid gene product of M. tuberculosis is unable to use PZA as substrate. Thus, as in *M. bovis* and *M.bovis* BCG, pncA is the only functional gene in *M. tuberculosis* coding for a functional PZAse. All strains of M.bovis reported till today are resistant to PZA due one single point mutation which completely inactivates the enzyme.

5 Summary

1. Rifampicin is one of the most potent and most effective drug against tuberculosis. Rifampicin resistance in the *M. tuberculosis* strain is caused due to mutation in *rpoB* gene, which in turn effects the RNA polymerase (RNAP). The enzyme complex as a whole is a well known target for new drug development. A simplified technology for the RNAP assay for the screening of drug was devised in the work. The method established was cost effective, suitable for high-throughput use, did not use radioisotopic material and utilized natural nucleotides. With the devised assay several drug candidates were tested.
2. Attempt was done to reconstitute *M. tuberculosis* RNAP. The subunits of *M. tuberculosis* RNAP viz. RpoA, RpoB, RpoC and RpoZ were individually cloned, expressed and purified and attempt was done to constitute core enzyme. However functional reconstitution was not achieved during the work.
3. Pyrazinamide (PZA) is a nicotinamide analog which is used as a frontline drug to treat tuberculosis. The exact mechanism of action of pyrazinamide (PZA), one of the most important antimycobacterial drug is still elusive. Mutations in *pncA* gene of *M. tuberculosis* are mostly responsible for the resistance developed by *M. tuberculosis* against PZA.
4. In other to further understand the molecular basis of PZA resistance in *M. tuberculosis*, DNA sequence of *pncA* from PZA resistant and susceptible clinical isolates of *M. tuberculosis* were analysed. The analysis identified 11 different as yet unreported mutations.
5. Further the pncA gene of *M. tuberculosis* H37Rv was cloned in *E. coli* BL21 (DE3) and purified up to homogeneity. Direct cell free PZase assay from the purified enzyme was performed using PZA as a substrate on the basis that POA, an end product of PZA reacts with Mohr's salt to produce orange-reddish product. A High throughput enzymatic Cell free PZase assay was developed in 96 well plate, which can be further used for the exploration of novel therapy for TB.
6. Thus, by the method described in this study quantitative results for pncA (PZAse) of a mycobacterial strain could be obtained within 2-3 days as compared to several weeks by currently standard methods.
7. In addition, the results confirm that amidD gene product of *M. tuberculosis* is unable to use PZA as substrate. Thus, as in *M. bovis* and *M.bovis* BCG, pncA is the only functional gene in *M. tuberculosis* coding for a functional PZAse. All strains of M.bovis reported till today are resistant to PZA due one single point mutation which completely inactivates the enzyme.

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7 Appendix

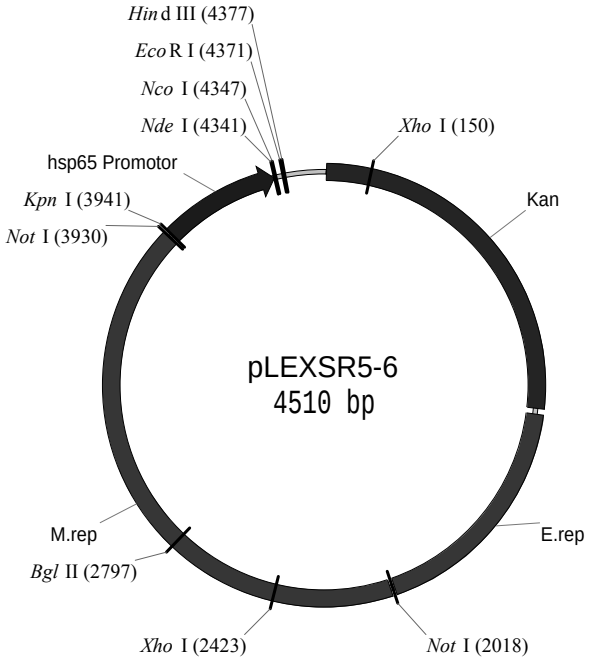
7.1 Abbreviations

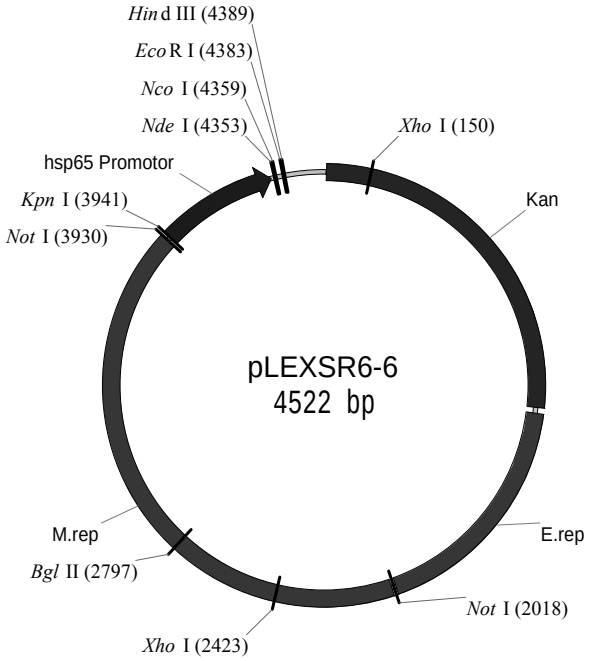
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin
bp	Base pair
BSA	Bovine Serum Albumin
CTP	Cytidine triphosphate
Dept.	Department
DNA	Desoxiribonucleic acid
dNTP	Desoxiribonucleoside triphosphate
ds	Double Stranded
EDTA	Ethylenediamine tetraacetic acid
g	Acceleration of gravity
GTP	Guanosine triphosphate
h	Hour
His	Histidine
HIV	Human immunodeficiency virus
hsp60	Heat shock protein 60
HTS	high-throughput screening
IPTG	Isopropyl-thio-β-Dgalactopyranoside
IS	Insertion Sequence
KAN	Kanamycin

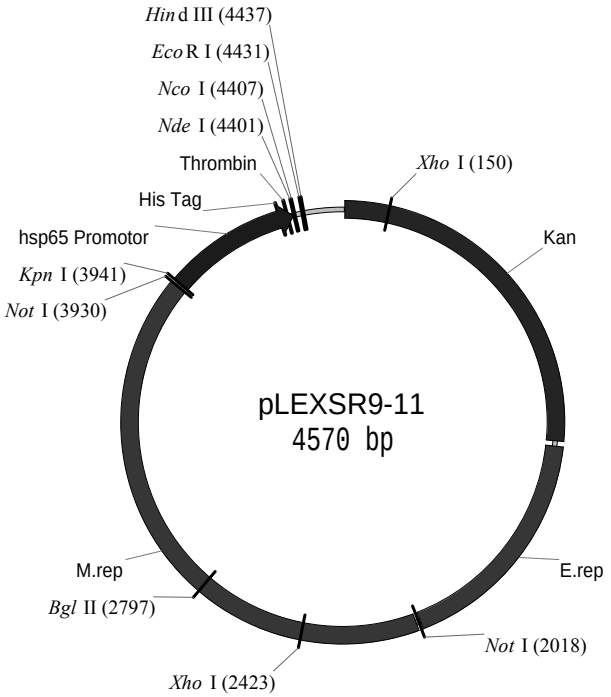
kb	Kilo base pair
kDa	Kilodalton
kV	Kilovolt
LB	Luria Bertani medium
M	Molar
Mb	Mega basepare
MDR	Multidrug resistant
MIC	Minimum Inhibiting Concentration
min	Minute
mM	Millimolar
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NTA	Nitrilotriacetic acid
NTP	Nucleoside triphosphate
OADC	Oleic acid, albumin, dextrose, catalase
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PBS	Phsphate buffered saline
PCR	Polymerase chain reaction
PEG	Poly ethylene glycol
POA	Pyrazoic acid

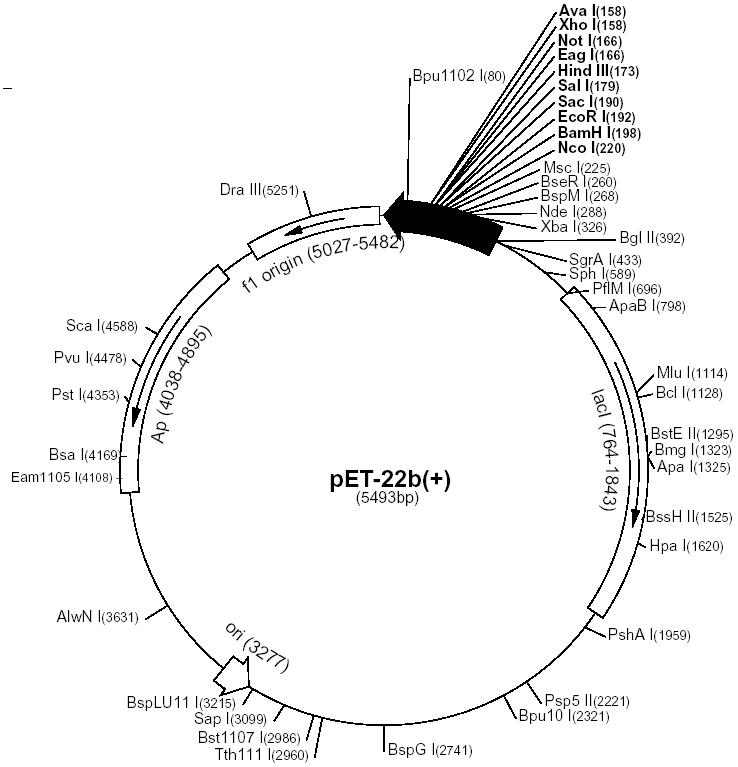
PVDF	Polyvinylidene fluoride
PZA	Pyrazinamide
PZase	Pyrazinamidase
R	Resistant
RLU	Relative light unit
RNA	Ribonucleic acid
RNAP	RNA polymerase
rpm	Rounds per minute
RT	Room temperature
s	Second
S	Susceptible
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TAE	Tris-Acetate-EDTA
TB	Tuberculosis
TEMED	N,N,N',N'-tetramethylethylenediamine
TTP	thymidine triphosphate
U	Unit
UTP	Uridine triphosphate
UV	Ultraviolet light
WHO	World Health Organization
XDR	Extreme Drug Resistance

7.2 Maps of plasmids






Hind III (4437)
Eco R I (4431)
Nco I (4407)
Nde I (4401)
Thrombin
His Tag
hsp65 Promotor
Kpn I (3941)
Not I (3930)
Xho I (150)
Kan
pLEXSR9-11
4570 bp
E.rep
Not I (2018)
Xho I (2423)
Bgl II (2797)
M.rep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Eag I(166)
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Sal I(179)
Sac I(190)
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BamH I(198)
Nco I(220)
Bpu1102 I(80)
Msc I(225)
BseR I(260)
BspM I(268)
Nde I(288)
Xba I(326)
Bgl II(392)
SgrA I(433)
Sph I(589)
PflM I(696)
ApaB I(798)
Dra III(5251)
f1 origin (5027-5482)
Sca I(4588)
Pvu I(4478)
Pst I(4353)
Ap (4038-4895)
Bsa I(4169)
Eam1105 I(4108)
pET-22b(+)
(5493bp)
Mlu I(1114)
Bcl I(1128)
lacI (764-1843)
BstE II(1295)
Bmg I(1323)
Apa I(1325)
BssH II(1525)
Hpa I(1620)
AlwN I(3631)
ori (3277)
PshA I(1959)
BspLU11 I(3215)
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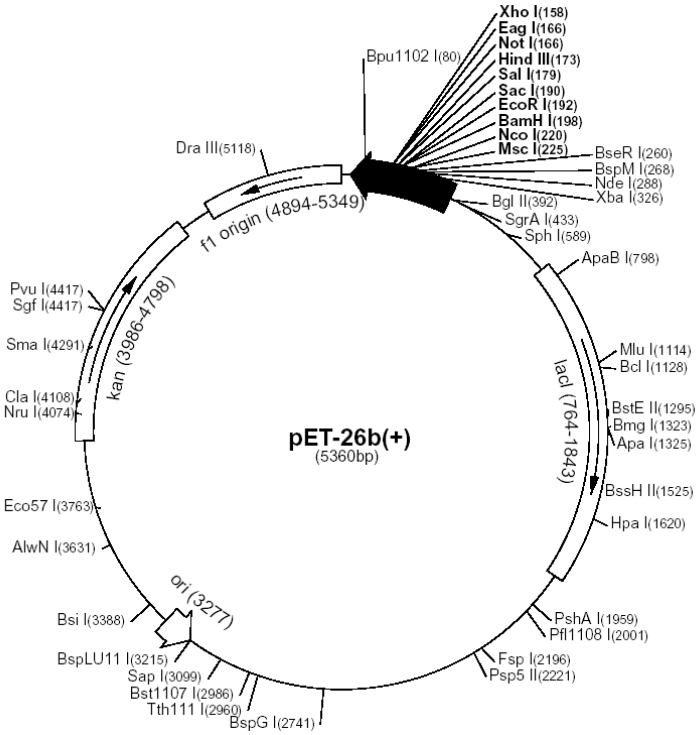
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Pvu I(4417)
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(5360bp)
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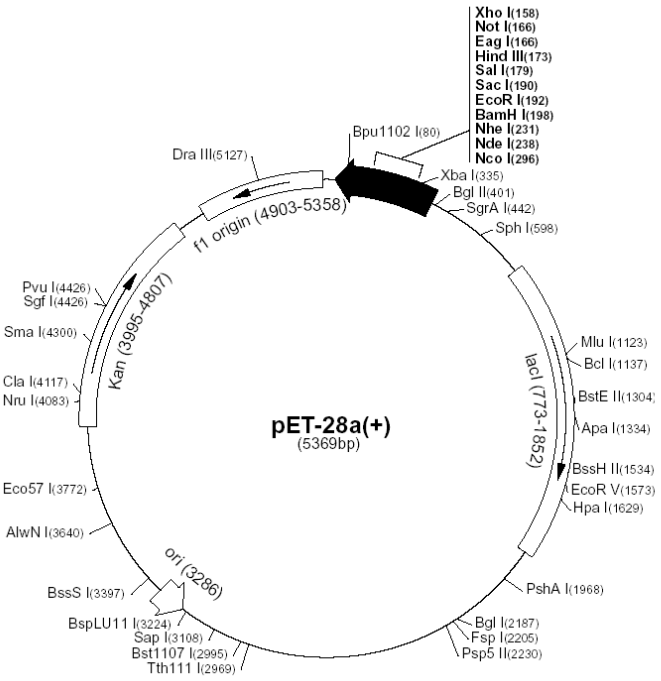
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SgrA I(442)
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Kan (3995-4807)
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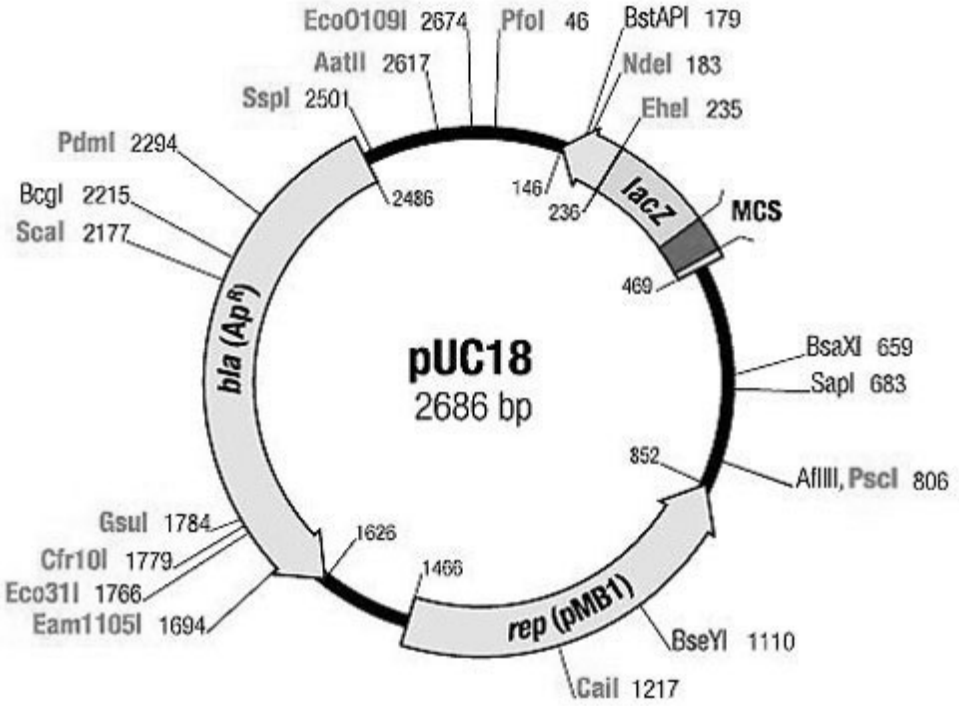
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BcgI 2215
ScaI 2177
2486
146
236
lacZ
MCS
469
bla (Ap^R)
BsaXI 659
SapI 683
852
AflIII, PscI 806
GsuI 1784
Cfr10I 1779
Eco31I 1766
Eam1105I 1694
1626
1466
rep (pMB1)
BseYI 1110
CaiI 1217